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EVALUACIÓN DEL EFECTO PROTECTOR DE UN EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ FRENTE AL ESTADO INFLAMATORIO PRESENTE EN EL PROCESO ATEROSCLERÓTICO

Memoria presentada por **Cristina Pérez Ternero** para optar al grado de Doctora en Farmacia, con Mención Internacional

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ABREVIATURAS

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	A
ACAT	Acil coenzima A:colesterol aciltransferasa *Acyl-CoA:cholesterol acyltransferase*
AIF	Factor de inducción de apoptosis *Apoptosis inducing factor*
ALT	Alanina aminotransferase *Alanine aminotransferase*
Apo	Apolipoproteína *Apolipoprotein*
ApoE-/-	Ratones ApoE deficientes *ApoE knockout mice*
AST	Aspartato aminotransferasa *Aspartate aminotransferase*
	B
BAEC	Células endoteliales de aorta bovina *Bovine aortic endothelial cells*
	C
CETP	Proteína de transferencia de esteres de colesterol *Cholesterylester transfer protein*
C_{max}	Concentración sérica máxima *Maximum serum concentration*
COX	Ciclooxigenasa *Ciclooxigenase*
CRP	Proteína C reactiva *C reactive protein*
CSA	Área transversal del vaso *Cross sectional área*
	E
EESA	Extracto enzimático de salvado de arroz
eNOS	Óxido nítrico sintasa endotelial *Endothelial nitric oxide synthase*

	F
Fis1	Proteína de fisión mitochondrial 1 *Mitochondrial fission 1 protein*
	G
G6pasa	Glucosa-6-fosfatasa *Glucose 6-phosphatase*
GPx	Glutation peroxidasa *Glutathione peroxidase*
	H
HED	Dosis equivalente en humanos *Human equivalent dose*
HDL	Lipoproteína de alta densidad *High-density lipoprotein*
HMG-CoA-Reductasa **HMG-CoA-Reductase**	3-hidroxi-3-metil-glutaril-CoA reductasa *3-hydroxy-3-methylglutaryl-CoA reductase*
	I
ICAM-1	Molécula de citoadhesión intracelular-1 *Intercellular adhesion molecule-1*
IDL	Lipoproteínas de densidad intermedia *Intermediate-density lipoprotein*
iNOS	Óxido nítrico sintasa inducible *Inducible nitric oxide synthase*
	L
LCAT	Lecitin colesterol acil transferasa *Lecithin–cholesterol acyltransferase*
LDL	Lipoproteínas de baja densidad *Low-density lipoprotein*
LDLR	Receptor de lipoproteínas de baja densidad *Low-density lipoprotein receptor*
LDLR-/-	Ratones deficientes en el receptor de la LDL *LDL receptor knockout mice*
	M
M-CSF	Factor estimulantes de colonias de macrófagos

	Macrophage colony-stimulating factor
Mfn1	Mitofusina-1 *Mitofusin-1*
Mfn2	Mitofusina-2 *Mitofusin-2*
MMP	Metaloproteinasa *Metalloproteinase*
MNC	Células mononucleares *Mononuclear cell*
MPO	Mieloperoxidasa *Myeloperoxidase*
	N
NADPHox	NADPH oxidasa *NADPH oxidase*
NO	Óxido nítrico *Nitric oxide*
NOAEL	Dosis sin efecto adverso observado *No observed adverse effect levels*
Nrf-1	Factor de respiración nuclear-1 *Nuclear respiratory factor-1*
Nrf-2	Factor de respiración nuclear-1 *Nuclear respiratory factor-1*
	O
$O_2^{\cdot-}$	Radical superóxido Superoxide radical
OMS	Organización Mundial de la Salud World Health Organization
Opa1	*Optic atrophy 1*
oxLDL	LDL oxidada *Oxidised LDL*
	P
PAI-1	Inhibidor del activador del plasminógeno-1 *Plasminogen activator inhibitor-1*

PBMC	Célula mononuclear de sangre periférica *Peripheral blood mononuclear cell*
PEPCK	Fosfoenolpiruvato carboxiquinasa *Phosphoenolpyruvate carboxykinase*
Pgc-1α	Cofactor 1α del receptor activado por el proliferador de peroxisomas *Peroxisome proliferator-activated receptor gamma coactivator 1α*
Pgc-1β	Cofactor 1β del receptor activado por el proliferador de peroxisomas *Peroxisome proliferator-activated receptor gamma coactivator 1β*
	R
RBEE	Rice bran enzymatic extract
ROS	Especies reactivas de oxígeno *Reactive oxygen species*
	S
SOD	Superóxido reductasa *Superoxide dismutase*
SRB1	Receptor scavenger clase B miembro1 *Scavenger receptor class B member 1*
SREBP	Proteína de unión al elemento regulador de esteroles *Sterol regulatory element-binding protein*
	T
TERT	Telomerasa transcriptasa inversa *Telomerase reverse transcriptase*
Tfam	Factor de transcripción mitocondrial A Mitochondrial transcription factor A
T_{max}	Tiempo para alcanzar la concentración sérica máxima *Time to reach maximum serum concentration*
	V
VCAM-1	Molécula de citoadhesión vascular-1 *Vascular cell adhesion molecule-1*
VLDL	Lipoproteínas de muy baja densidad Very low-density lipoprotein
	X

XO	Xantina oxidase *Xanthine oxidase*

INTRODUCCIÓN

1. EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ (EESA)

El arroz (*Oryza sativa*) es uno de los cereales más consumidos a nivel mundial junto con el maíz y el trigo. Su producción mundial alcanza los 749.7 millones de tonelada (FAO, 2016), siendo sus mayores productores China, India, Indonesia y Pakistán (FAO). Se conocen cerca de diez mil variedades de arroz, que se encuadran dentro de dos subespecies: la variedad Índica, de grano largo y la variedad Japónica, de grano corto.

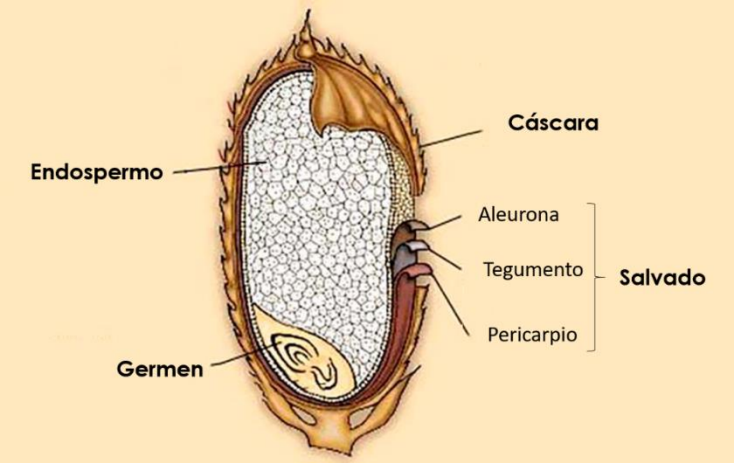

Figura 1: Partes del grano de arroz. Modificado de Encyclopaedia Britanica, Inc., 1996.

Del exterior hacia adentro, el **grano de arroz se compone de** (i) la cáscara (20%) que impermeabiliza y protege de los agentes externos medioambientales; (ii) el salvado (6%), que a su vez se compone del pericarpio (cubierta más externa de la semilla), el tegumento (membrana dura de protección) y la aleurona (rica en proteínas); (iii) el germen (2%); y (iv) el endospermo (72%), rico en almidón **(Figura 1)** (Islam et al., 2011). El elevado consumo de arroz se justifica por ser el endospermo una fuente de excelente hidratos de carbono a un bajo coste de producción. Sin embargo, durante el proceso de refinado del arroz se retira la capa del **salvado**, que constituye la principal fuente de micronutrientes y moléculas bioactivas presentes en el arroz y que le confieren su verdadero potencial nutricional, al margen del valor energético.

En la **fracción insaponificable** del salvado de arroz se encuentran en una elevada concentración micronutrientes con interesantes propiedades para la promoción de la salud, como el γ-oryzanol (una mezcla de esteres del ácido ferúlico con alcoholes

triterpénicos o fitosteroles), el ácido ferúlico, los fitosteroles y la vitamina E (tocoferoles y tocotrienoles), entre otros (Jariwalla, 2011) **(Figura 2)**.

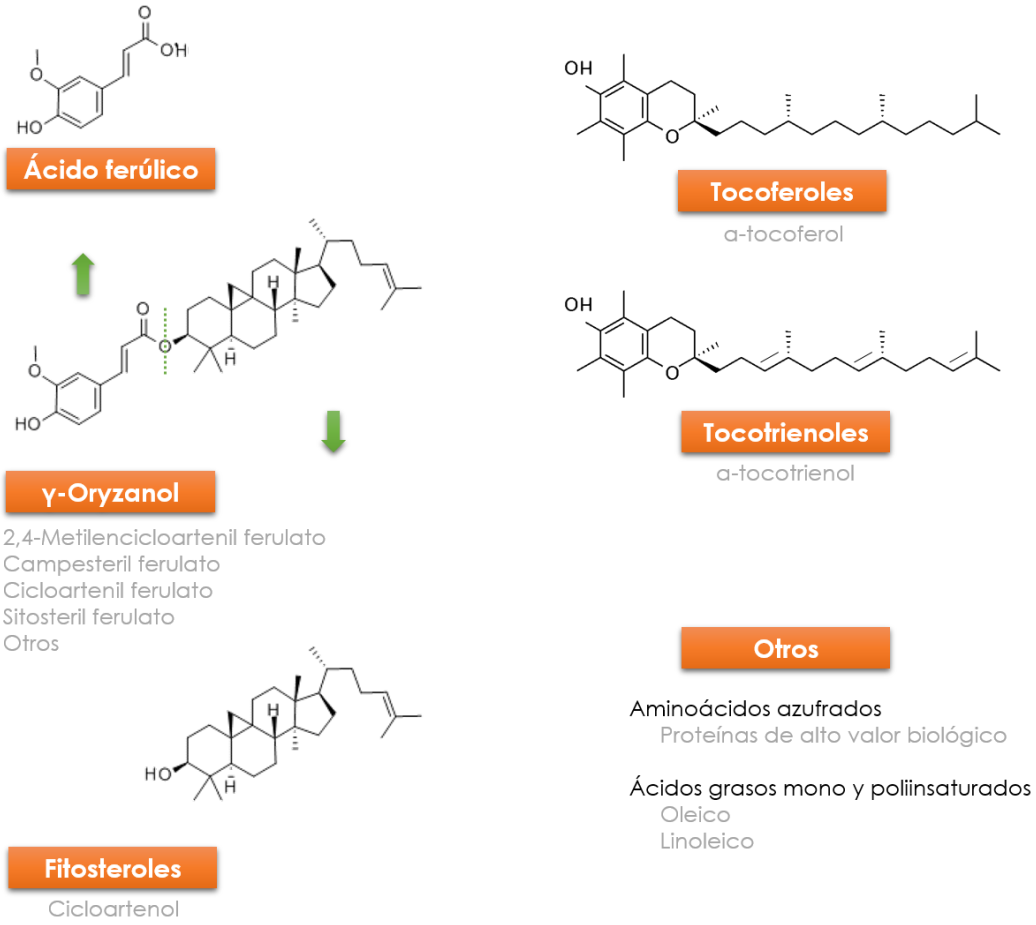

Figura 2: Estructura química de las principales moléculas bioactivas en el salvado de arroz.

Además, el salvado de arroz presenta una composición en macronutrientes superior a la del grano refinado de arroz, el cual contienen una elevada cantidad de carbohidratos (74-82%), mientras que el salvado contiene un 11-13% de proteínas de alto valor biológico, un 11-12% de fibra insoluble, un 15-20% de grasas y sólo un 40-45% de hidratos de carbono.

Durante el proceso de refinado del arroz blanco se producen grandes cantidades de salvado de arroz. Sin embargo, a pesar de su potencial biológico, el salvado de arroz termina siendo desechado o es destinado a la alimentación del ganado. Esta aparente contradicción se explica por la **naturaleza conjugada** de muchas de las moléculas

bioactivas, lo cual limita su biodisponibilidad, por la presencia de **antinutrientes** (inhibidores de tripsina, fitatos y lectina) (Khan et al., 2009) y por los efectos de **lipasas intrínsecas**, que hacen del salvado de arroz un producto inestable y por lo tanto de bajo interés para la industria alimentaria humana. El rápido enranciamiento de los componentes lipófilos confiere al salvado de arroz un sabor amargo y da lugar a productos de reacción que pueden suponer un riesgo toxicológico, impidiendo su consumo seguro por la población. Todas estas características hacen necesarios los procesos de **extracción y estabilización**. La presentación más común derivada del salvado de arroz es en forma de aceite, la cual permite la solubilización y concentración de la fracción insaponificable.

Los métodos más eficientes de extracción y estabilización son aquellos basados en la extracción físico/química que emplean disolventes orgánicos como el hexano o diversas técnicas como irradiación infrarroja, liofilización, autoclavado, adición de antioxidantes o tostado, o la extracción asistida con fluidos supercríticos, por calentamiento óhmico o por microondas, entre otros (Oliveira et al., 2012; Yılmaz et al., 2014; Yılmaz et al., 2015; Goufo et al., 2015). Estas técnicas cuentan con numerosas **desventajas**: (i) existe el riesgo de la presencia de trazas si se han empleado disolventes orgánicos, lo cual impedirían su consumo humano, (ii) se llevan a cabo en condiciones extremas a altas temperaturas, lo que supone una pérdida de compuestos bioactivos, (iii) presentan problemas de conservación debido a que no se resuelve la tendencia a la rápida degradación de los ácidos grasos.

Dadas las carencias que presentan los métodos tradicionales de extracción, el grupo de Tecnología y Aplicación de Enzimas del Departamento de Bioquímica y Biología Molecular de la Universidad de Sevilla, desarrolló en 2006 un método de extracción y estabilización para el salvado de arroz por medio de **hidrólisis enzimática** (Parrado et al., 2006). A diferencia de los métodos tradicionales, la hidrólisis enzimática garantiza la ausencia de componentes xenobióticos o tóxicos y protegen la integridad de los componentes bioactivos, pues la extracción se lleva a cabo en condiciones de pH neutro y temperatura moderada. Además, la extracción enzimática consigue la hidrólisis de las proteínas en pequeños péptidos o aminoácidos, y de moléculas activas, que estaban inicialmente conjugadas, haciéndolos solubles y bioaccesibles. La interacción de estos aminoácidos con sustancias hidrófobas, como son las grasas y otros componentes lipófilos de la fracción insaponificable del salvado, también permiten su solubilización. Finalmente, la inactivación de las lipasas endógenas del salvado evita el enranciamiento de los ácidos grasos y pérdidas de otros componentes esenciales como los aminoácidos u otros micronutrientes como el γ-oryzanol.

Desde el punto de vista **cuantitativo**, el proceso de extracción enzimática supone un incremento en la concentración de las moléculas bioactivas de la fracción insaponificable. Este tipo de extracción aporta un cambio cualitativo en la proporción de los macronutrientes en favor de las proteínas. En el perfil aminoacídico destacan la abundancia de L-arginina, que posee un efecto vasodilatador por ser precursora de óxido nítrico, y los aminoácidos azufrados (cisteína y metionina), a los que se atribuyen propiedades antioxidantes ya que el grupo SH de su molécula participa en reacciones redox y además son precursores de antioxidantes naturales como el glutatión y la taurina. El extracto enzimático de salvado de arroz también es rico en minerales (Ca, Mg, Fe) y vitaminas del grupo B (B_1, B_2, B_6, B_{12}) y presenta un interesante perfil de ácidos grasos rico en ácidos grasos monoinsaturados (ω9, oleico, 41.72 ± 4.14% del total de ácidos grasos) y poliinsaturados (ω6, linoleico, 35.00 ± 0.12% del total de ácidos grasos; ω3, linolenico, 0.76 ± 0.28% del total de ácidos grasos) (Parrado et al., 2006).

2. BIODISPONIBILIDAD DE LOS PRINCIPALES COMPUESTOS ACTIVOS EN EL EESA

El salvado de arroz es una excelente fuente de múltiples compuestos bioactivos para la prevención de enfermedades cardiovasculares. Para una mejor compresión de los mecanismos moleculares que subyacen a estos efectos, resulta de vital importancia esclarecer qué compuestos son absorbidos y en qué magnitud, así como los factores que afectan a dicha absorción. El termino **biodisponibilidad** alude a la cantidad de una molécula que alcanza circulación sistémica y está gobernada por las propiedades químicas intrínsecas de las moléculas y de la presencia de otras sustancias en el tracto digestivo, así como de características del individuo (edad, sexo, patologías…).

Una de las moléculas presentes en el salvado de arroz que más interés ha despertado por sus propiedades beneficiosas es el **γ-oryzanol**. El γ-oryzanol es una mezcla de hasta 10 esteres del ácido ferúlico con diferentes esteroles o alcoholes triterpénicos, lo que le confiere una baja hidrosolubilidad, necesaria para la absorción gastrointestinal.

Numerosos grupos han intentado describir su absorción. Sin embargo, ningún estudio hasta el momento ha reportado la presencia de la molécula de γ-oryzanol intacta en sangre (Gillespie, 2003; Lubinus et al., 2013) debido a que la penetración de γ-oryzanol a través de la barrera intestinal es muy baja (<0.5%) (Zhu et al., 2015). Este hecho se ha confirmado por su elevada recuperación en heces (Gillespie, 2003; Lubinus et al., 2013). Además, estudios de bioaccesibilidad (fracción máxima liberada de la matriz de un alimento en el tracto gastrointestinal) de estéril ferulatos presentes en el salvado de arroz

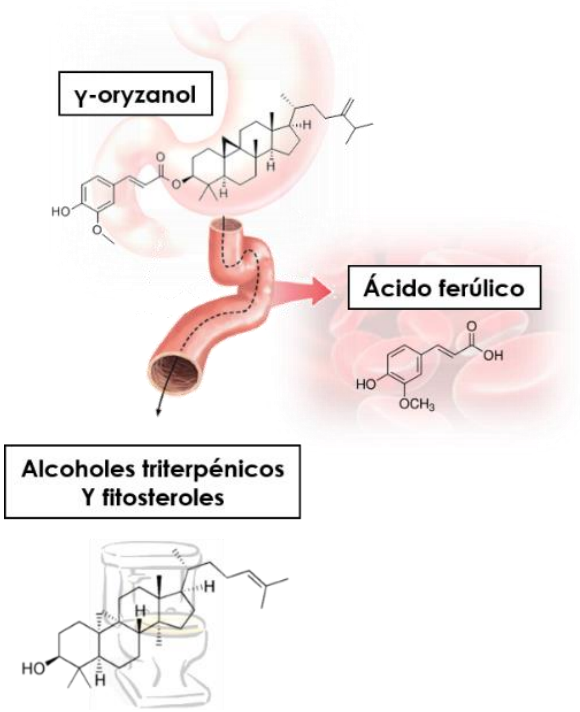

Figura 3: Esquema de la hidrólisis de γ-oryzanol en el intestino, que da lugar a ácido ferúlico, que será absorbido y tendrá efectos sistémicos, y a alcoholes triterpénicos y fitosteroles que ejercerán sus acciones a nivel intestinal

apenas muestran incremento alguno de γ-oryzanol libre tras someter a la muestra a una digestión *in vitro*. Sin embargo, sí disminuye su concentración como forma asociada a la matriz, al tiempo que el contenido de esteroles libres aumenta a más del doble. Estas observaciones se pueden relacionar con una **rápida hidrólisis** del enlace éster por parte de esterasas pancreáticas y de microorganismos presentes en el tracto intestinal (Berger et al., 2005; Mandak et al., 2012). La teoría de la rápida hidrólisis del γ-oryzanol seguida de la absorción de la molécula de ácido ferúlico se ha confirmado por varios estudios en modelos animales en los que se ha identificado la presencia de ácido ferúlico en plasma tras la administración de γ-oryzanol **(Figura 3)** (Fujiwara et al., 1982; Fujiwara et al., 1983; Pan et al., 2014).

El **ácido ferúlico** es un polifenol perteneciente a la familia del ácido hidroxicinámico. Se encuentra ampliamente distribuido en la naturaleza, soluble en forma libre o conjugado a azúcares u otras pequeñas moléculas, o bien en forma insoluble, esterificado con arabinoxilanos (Zhao et al., 2008). El ácido ferúlico es especialmente abundante en el salvado de cereales, donde se encuentra fundamentalmente en su forma

insoluble. Mientras que la forma libre es absorbida de forma muy eficiente, las formas conjugadas requieren la hidrólisis del enlace éster previo a la absorción en el intestino delgado (Kern et al., 2003; Adam et al., 2003). Dicha absorción, al igual que su metabolismo y eliminación, se producen de forma muy rápida, alcanzando t_{max} de entre 15 minutos y 2 horas y siendo prácticamente indetectable en plasma pasadas 24 h (Fujiwara et al., 1982; Fujiwara et al., 1983; Manach et al., 2005; Pan et al., 2014).

Los **fitosteroles** (esteroles y estanoles) son moléculas hidrofóbicas que tienden a formar cristales estables, que deben ser solubilizados o emulsificados en las micelas mixtas intestinales para ser biodisponibles y ejercer sus acciones biológicas (Ostlund et al., 1999). La absorción intestinal de esteroles y estanoles es muy baja y está mediada por un transportador localizado en la pared del enterocito, denominado NPC1L1 (Turley, 2008). Si bien la absorción de esteroles es muy baja, la de estanoles es aún menor. Así, esteroles como el campesterol y el sitosterol tienen valores de biodisponibilidad de 1.9 y 0.5% respectivamente, mientras que para el sitostanol es de 0.04% (Ostlund et al., 2002). Esta baja absorción se debe a que cuando las micelas entran en contacto con las microvellosidades y penetran en el enterocito, los fitosteroles tienen una alta afinidad por los transportadores ATP-dependientes de tipo ABCG5/8, localizados en la membrana apical del enterocito, que los devuelve a la luz intestinal, de manera que existe un reciclaje y la absorción neta es muy baja (Brauner et al., 2012). El bajo porcentaje de esteroles que llegan al torrente sanguíneo son esterificados en el plasma y transportados al hígado por medio de las lipoproteínas de alta densidad (HDL) (Rossi et al., 2002).

El término **vitamina E** comprende a 4 tocoferoles y 4 tocotrienoles que se diferencian en las insaturaciones presentes en la cadena lateral de los tocotrienoles. Comparado con los tocoferoles, los tocotrienoles se encuentran en menor proporción en la naturaleza. Sin embargo las insaturaciones de la cadena lateral les confiere un mayor carácter lipófilo que permite una distribución más eficaz en las membranas celulares, una mayor movilidad y permeación en tejidos grasos como el hígado o el cerebro y una mayor potencia antioxidante **(Figura 4)** (Suzuki et al., 1993; Atkinson et al., 2008). Para la absorción de ambas formas de vitamina E son necesarios procesos de emulsión y lipolisis en la luz intestinal mediante sales biliares y enzimas pancreáticas (Traber, 2013). Sin embargo, se han descrito mecanismos diferenciados para tocoferoles y tocotrienoles en lo que respecta a la absorción y transporte por el organismo. Mientras que los tocotrienoles son transportados en partículas de HDL, los tocoferoles son preferentemente transportados en quilomicrones, lo cual determina la distribución preferente de tocotrienoles en tejidos grasos mientras que los tocoferoles son distribuidos en base a los receptores de LDL (LDLR), presentes en todos los tejidos (Gee, 2011a). Además, los tocotrienoles tienen una baja afinidad por la proteína

transportadora de α-tocoferol, lo cual puede explicar la rápida desaparición de los tocotrienoles pasadas 24 h tras su ingestión (Gee, 2011b).

Figura 4: Diferente distribución de tocoferoles y tocotrienoles en las membranas celulares.

3. ACTIVIDAD BIOLÓGICA DEL SALVADO DE ARROZ

El salvado de arroz contiene una **multitud de moléculas bioactivas** que actúan de forma sinérgica y sincrónica. Si bien la actividad hipocolesterolemiante es la más reconocida, el salvado de arroz posee un elevado potencial antioxidante, antiinflamatorio y regulador del metabolismo de la glucosa. Estas actividades se discutirán en las siguientes secciones, así como otras actividades no relacionadas con el modelo de aterosclerosis estudiado en la tesis como la actividad anticancerosa o las aplicaciones en cosmética derivada del potencial antioxidante.

a) Actividad hipocolesterolemiante

Numerosos estudios epidemiológicos respaldan la relación directa entre los niveles de colesterol plasmático y la incidencia de eventos cardiovasculares. Aunque existen fármacos que consiguen una reducción eficaz del colesterol plasmático, las guías en prevención recomiendan la corrección de los hábitos y esquemas alimentarios como estrategias de base, entrando en juego los alimentos funcionales como el salvado de arroz, ricos en moléculas con potencial hipocolesterolemiante.

El **mecanismo de la acción hipocolesterolemiante** del salvado de arroz no se conoce en toda su dimensión debido a que el salvado contiene un gran número de moléculas potencialmente activas. Sin embargo, no parece que este efecto se deba a una única molécula, ni que esté mediado por un único mecanismo. La hipótesis más barajada sostiene que los efectos observados se deben a la interacción de los polifenoles, γ-oryzanol, fitosteroles y tocotrienoles en los procesos de absorción, transporte, metabolismo y síntesis del colesterol. Además, cabe destacar que aunque la composición del salvado de arroz tiene un perfil de ácidos grasos mono- y poli-insaturados muy beneficioso, la bajada de colesterol plasmático asociada al consumo de salvado de arroz no estaría justificada por el perfil de ácidos grasos del mismo, sino por los compuestos que se encuentran en fracción insaponificable (γ-oryzanol, fitosteroles y tocotrienoles) (Rong et al., 1997).

Una de las moléculas presentes en el salvado que recaba mayor atención es el **γ-oryzanol**. Son muchos los estudios que reportan como principal mecanismo del γ-oryzanol, un incremento en la secreción biliar y en la excreción fecal de esteroles en su forma libre y esterificada (Cicero et al., 2001). Los efectos del γ-oryzanol son mediados de forma sinérgica por las dos moléculas que lo conforman: ácido ferúlico y fitosteroles o alcoholes triterpénicos.

Así, tanto el consumo de **γ-oryzanol** como de **ácido ferúlico** han mostrado mejoras en los niveles de colesterol total, LDL (lipoproteínas de baja densidad), VLDL (lipoproteínas de muy baja densidad) y HDL (Wilson et al., 2007) por medio de la reducción de la actividad de β-hidroxi-β-metil-glutaril-CoA reductasa (HMG-CoA-Reductasa) (Wang et al., 2015) o de la expresión de SREBP-1/2 (Sterol regulatory element-binding protein), necesarios para la transcripción de HMG-CoA-Reductasa (Wang et al., 2015; Naowaboot et al., 2016). Resulta interesante que a igual dosis, el γ-oryzanol obtiene mejores resultados sobre la reducción de colesterol LDL y VLDL comparado con el ácido ferúlico, debido al efecto adicional a nivel intestinal de la mitad esteroídica del γ-oryzanol que no comparte el ácido ferúlico (Wilson et al., 2007).

A diferencia del ácido ferúlico, el γ-oryzanol reduce la absorción de colesterol intestinal, en un proceso llevado a cabo por medio de los fitosteroles o alcoholes triterpénicos que forman parte de su molécula, como puso de manifiesto un estudio en el que la suplementación de una dieta baja en colesterol con un 0.5% de γ-oryzanol no supuso ningún cambio en el flujo biliar o en su composición. Sin embargo, cuando la suplementación con γ-oryzanol se hizo en una dieta alta en colesterol, se incrementó el flujo biliar y la excreción de colesterol en un 12% y 28%, respectivamente (Seetharamaiah et al.,

1990), indicando el importante papel de los fitosteroles a nivel intestinal sobre el colesterol de la dieta.

Existe un gran consenso sobre la actividad hipocolesterolemiante de los **fitosteroles**. El consumo diario de 2 g de fitosteroles está asociado a una reducción del colesterol LDL de hasta el 15%. En el salvado de arroz se encuentran presentes tres tipos de fitosteroles: 4,4'-dimetilesteroles, 4-monometilesteroles y 4-desmetilesteroles (Sayre et al., 1990). Estructuralmente, los esteroles vegetales difieren del colesterol en la presencia de sustituyentes de tipo metilo o etilo en la cadena lateral de la molécula y en ocasiones de insaturaciones, lo cual les confiere un carácter más lipófilo, comparado con el colesterol. Estas diferencias estructurales son las responsables del efecto hipocolesterolémiante de los esteroles y de su baja absorción a nivel intestinal.

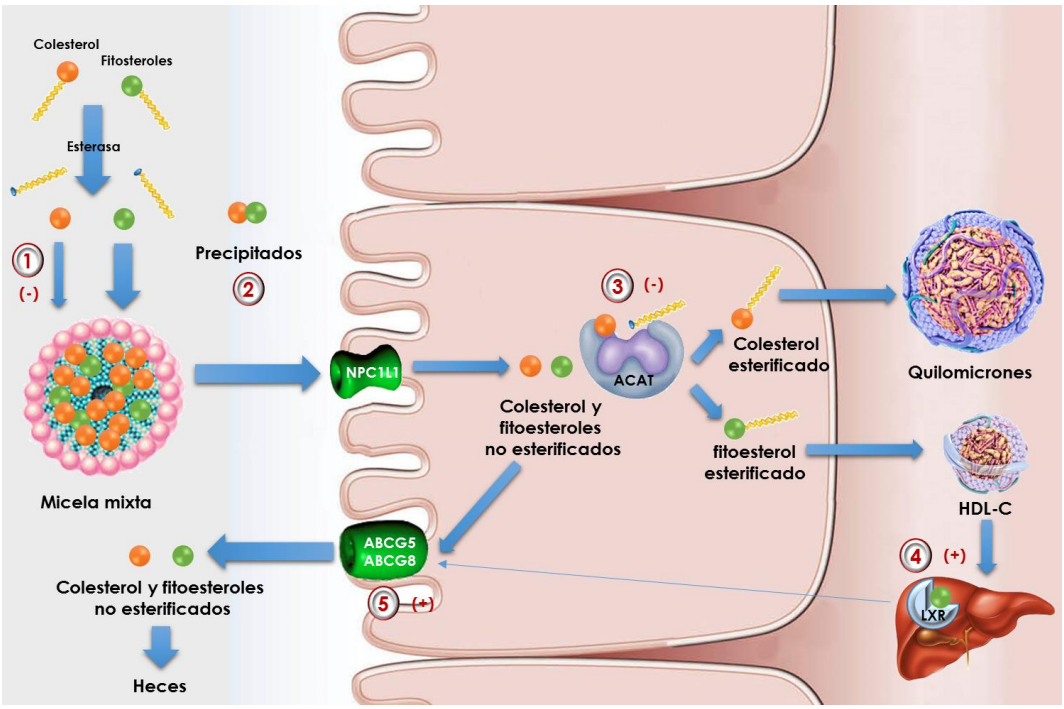

Figura 5: Mecanismos hipocolesterolemiantes a nivel intestinal de los fitosteroles. (1) Desplazamiento de las micelas mixtas. (2) Precipitación del colesterol. (3) Inhibición de la enzima acil CoA colesterol-acil transferasa (ACAT). (4) actividad agonista sobre el receptor hepático LXR. (5) Incremento en la expresión de los transportadores ABCG5/8.

En primer lugar, dada su mayor lipófila, los esteroles desplazan competitivamente al colesterol de las micelas mixtas formadas en la luz intestinal por las sales biliares y los

fosfolípidos, que es necesario para el transporte intraluminal hasta las microvellosidades intestinales (Ikeda et al., 1988). Además, se conoce que los esteroles son capaces de precipitar el colesterol en la luz intestinal, impidiendo su absorción (Ikeda et al., 1988; Christiansen et al., 2001). Adicionalmente, una vez en el enterocito, los esteroles inhiben la reesterificación del colesterol por acil CoA colesterol-acil transferasa (ACAT), necesaria para el transporte de colesterol en los quilomicrones, con lo cual, el colesterol no reesterificado es secretado de nuevo al lumen intestinal (Krause et al., 1993; Trautwein et al., 2003). Finalmente, los esteroles incrementan la expresión de los transportadores de tipo ABCG5/8 en la membrana apical del enterocito, acelerando el flujo de colesterol desde el enterocito hacia el lumen intestinal (Berge et al., 2000; Repa et al., 2002). Además del efecto directo en el enterocito, la actividad agonista de los esteroles sobre el receptor nuclear LXR hepático, estaría relacionada con la estimulación de la expresión de sus genes diana ABCG5/8 y ABCA1 (Janowski et al., 1996; Plat et al., 2005). Todos estos mecanismos contribuyen al reciclaje de los fitosteroles, prolongando su efecto en el organismo, y lo que es más interesante, se obtiene un efecto inhibitorio sobre la absorción del colesterol intestinal, tanto del proveniente de la dieta como del biliar, que será excretado con las heces, consiguiendo una reducción del colesterol LDL sin afectar al HDL **(Figura 5)**.

Finalmente, numerosos estudios en modelos animales y ensayos clínicos respaldan la actividad hipocolesterolémiante de los tocotrienoles. Administrados por la vía intravenosa, inhiben la actividad y expresión de la enzima HMG-CoA-Reductasa por dos vías: i) acelerando su degradación y ii) reduciendo la eficacia de transcripción del ARN que codifica al enzima (Parker et al., 1993). Además, las diferencias estructurales entre tocoferoles y tocotrienoles parecen ser esenciales para la inhibición efectiva del enzima HMG-CoA-Reductasa (Iqbal et al., 2003) **(Figura 6)**.

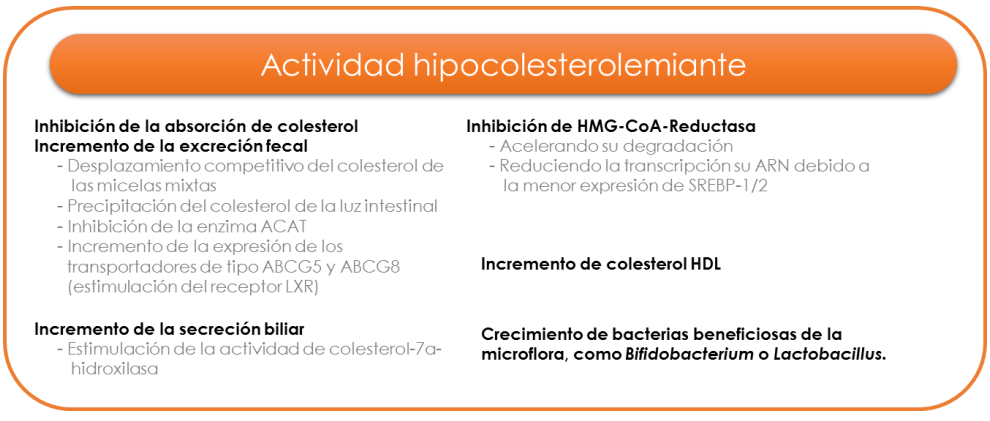

Figura 6: Principales mecanismos de la acción hipocolesterolemiante.

b) Actividad antioxidante

Existe un interés creciente por productos naturales ricos en moléculas antioxidantes que protejan frente a procesos degenerativos y enfermedades, que como las cardiovasculares, inflamatorias, el cáncer, y el envejecimiento, estén causadas por el daño oxidativo de los radicales libres de oxígeno (RLO) en el ADN y proteínas. El salvado de arroz contiene una considerable cantidad de moléculas con actividad antioxidante. De entre ellas destacan el **γ-oryzanol y la vitamina E**, y en menor medida una amplia variedad de polifenoles (Goufo et al., 2014). Los mecanismos responsables de esta actividad están relacionados con el incremento de las defensas antioxidantes **(incremento de la expresión de enzimas antioxidantes** como la superóxido dismutasa (SOD), catalasa, glutatión peroxidasa (GPx) o glutatión reductasa) o con la **reducción de las fuentes de estrés oxidativo** (reducción de la expresión proteica de las subunidades $p22^{phox}$, $p47^{phox}$ y Nox-1de la enzima NADPH oxidasa (NADPHox)). Esta protección del salvado de arroz frente a la producción de radicales libres reduciría la oxidación del colesterol presente en las membranas celulares, lo cual juega un papel importante en el inicio y progreso de la aterosclerosis (Lopez-Revuelta et al., 2005).

La actividad antioxidante del **γ-oryzanol es equiparable a la del ácido ferúlico** no esterificado (Nyström et al., 2005; Srinivasan et al., 2007). Esto indica que debe ser la molécula de ácido ferúlico la responsable de los efectos antioxidantes atribuidos a la molécula de γ-oryzanol. Sin embargo, existen estudios que también reportan actividad antioxidante de la mitad esteroidea, pero únicamente en sistemas vivos y no en aquellos libres de células (Islam et al., 2009). Este hecho descarta la neutralización directa de los radicales libres como mecanismo principal, y por tanto la actividad antioxidante se debe a la inhibición de la producción de radicales libres de oxígeno a través de enzimas como NADPHox y xantin oxidasa (XO) en células vivas (Islam et al., 2009).

La **aplicación cosmética** del aceite de salvado de arroz y del γ-oryzanol ha ido creciendo en los últimos años, siendo objeto de mejoras tecnológicas para incrementar su biodisponibilidad, como su inclusión en niosomas o liposomas (Viriyaroj et al., 2009; Bernardi et al., 2011; Manosroi et al., 2012). Dada su capacidad antioxidante se ha empleado para la conservación y estabilización de los preparados cosméticos (Juliano et al., 2005) o bien por sus propiedades emulgentes, reestructurantes y contra los radicales libres para frenar el envejecimiento cutáneo o en bloqueadores solares (Santa-Maria et al., 2010; Manosroi et al., 2012).

La **vitamina E** es ampliamente conocida por su actividad antioxidante, basada en el sistema redox tocoferol-tocoferilquinona, que le permite ser secuestrador de radicales

libres de oxígeno y radicales peróxidos (Newaz et al., 1999; Newaz et al., 2003; Kanaya et al., 2004). La cadena lateral presente en tocoferoles y tocotrienoles otorga un carácter lipófilo adecuado para su distribución en las membranas celulares, desde donde ejercen su actividad antioxidante. Cabe destacar que la insatauración de la cadena lateral presente en los tocotrienoles confiere una mayor fluidez a las membranas, y facilita la distribución de los tocotrienoles, lo cual puede estar detrás del hecho de que los tocotrienoles sean del orden de 40-60 veces más antioxidantes que sus análogos tocoferoles (Xu et al., 2001; Deepam et al., 2011) **(Figura 7)**.

Figura 7: Principales mecanismos de la acción antioxidante.

c) Actividad antiinflamatoria

El proceso inflamatorio forma parte de los mecanismos de defensa del organismo, pero si se prolonga en el tiempo, puede llevar al daño tisular. Además, durante el proceso inflamatorio las células inmunes son activadas, incrementando la liberación de RLO.

El factor de transcripción **NF-κβ** es un complejo proteico que controla la transcripción de genes implicados en el proceso inflamatorio. Ante un estímulo, como los RLO o citoquinas proinflamatorias como el TNF-α o la IL-1β, la enzima quinasa Ικκ se activa y fosforila a la proteína inhibitoria Ικβα, lo cual supone su degradación por el proteosoma. Como consecuencia, ocurre la activación y translocación al núcleo del heterodímero p50-p65, con la consiguiente trascripción de genes implicados en el proceso inflamatorio. Dado que los RLO son un estímulo proinflamatorio, la actividad antiinflamatoria derivada del salvado de arroz podría deberse en parte a la capacidad antioxidante del mismo, aunque también existe un efecto directo sobre el complejo del NF-κβ y otros mediadores de su activación.

Existen estudios que reportan la capacidad inhibitoria sobre la activación del NF-κβ del **γ-oryzanol** y de polifenoles del grupo del ácido hidroxicinámico como el **ácido ferúlico** (Akihisa et al., 2000; Nagasaka et al., 2007; Islam et al., 2008; Islam et al., 2009). Esta inhibición tendría como resultado una menor expresión de enzimas proinflamatorias como la óxido nítrico sintasa inducible (iNOS), ciclooxigenasa 2 (COX-2) o mieloperoxidasa (MPO), metaloproteinasas (MMP), de citoquinas proinflamatorias (TNF-α, IL-1β, PGE_2), o de la producción de óxido nítrico (NO), como se ha demostrado tras el consumo de diferentes preparados con salvado de arroz (Shalini et al., 2012; Huang et al., 2005) **(Figura 8)**.

Figura 8: Principales mecanismos de la acción antiinflamatoria.

La activación del NF-κβ, que está relacionada con los procesos inflamatorios implicados en las fases tardías de los **procesos alérgicos**.

Un estudio con dietas enriquecidas en **aceite de salvado de arroz** o aceite de salvado de arroz enriquecido con **γ-oryzanol** señala un incremento de la respuesta inmune debida a proliferación de linfocitos B y citoquinas derivadas de Th1 como IL-2 o TNF-α, mientras que aquellas derivadas de linfocitos Th2 (IL-4 y IgE), asociadas con propiedades antiinflamatorias, se redujeron (Sierra et al., 2005). Sin embargo, otro estudio con un extracto de salvado de arroz consiguió una menor presencia de leucocitos y de la degradación de Iκβα (Min et al., 2010).

Otros estudios con **γ-oryzanol realizados por la vía tópica**, o *in vitro* apuntan a efectos antialérgicos (Grassi et al., 2000; Juliano et al., 2005; Oka et al., 2010). Estos efectos antialérgicos se pusieron de manifiesto con el uso de diferentes preparados de salvado de arroz mediante la reducción de la respuesta inflamatoria y la liberación de citoquinas proinflamatorias (Choi et al., 2010), la atenuación de la degranulación de mastocitos por medio del bloqueo de la IgE (Oka et al., 2010; Fang et al., 2012; Mizushina et al., 2013) o la inhibición de la liberación de histamina y β-hexosaminidinasa (Iuo et al., 2005).

d) Actividad reguladora del metabolismo de la glucosa

Las dietas ricas en cereales integrales han demostrado su capacidad para reducir el riesgo de desarrollar diabetes en un 20-30% gracias a su contenido en fibra y en moléculas bioactivas (Belobrajdic et al., 2013).

Numerosos estudios en modelos animales proponen que suplementos de **γ-oryzanol** con la dieta reducirían los niveles de glucosa en ayunas, mientras que el consumo de ácidos hidroxicinámicos incrementaría la secreción de adiponectina, implicada en la sensibilidad de los tejidos a la insulina, mediante la inhibición de la activación del NF-κβ (Ohara et al., 2009). El γ-oryzanol también se ha relacionado con la regulación de la secreción de la insulina por el páncreas y del metabolismo de la glucosa mediante la normalización de la actividad de enzimas hepáticas (Son et al., 2011; Ghatak et al., 2012; Kozuka et al., 2013). Por otra parte, el **ácido fítico** ha conseguido reducir la hiperglicemia elevando la actividad de glucoquinasa hepática y reduciendo la de fosfoenolpiruvato carboxiquinasa (PEPCK) y de glucosa-6-fosfatasa (G6pasa) (Kim et al., 2010). Además, los **tocotrienoles** presentes en el salvado de arroz actúan como moduladores de los factores de transcripción PPAR (peroxisome proliferator-activated receptor), que regulan la expresión de genes implicados en el metabolismo lipídico y de carbohidratos (Ohara et al., 2009). La unión de tocotrienoles a PPAR favorece la expresión del transportador de glucosa 4, que promueve la acción de la insulina (Siddiqui et al., 2010).

No sólo el consumo de las moléculas aisladas ha demostrado efectos antidiabéticos, sino que un estudio llevado a cabo con **salvado de arroz** en ratones diabéticos, también consiguió normalizar los niveles de glucosa plasmática mediante la restauración de la actividad de glucoquinasa hepática y de la capacidad de almacenamiento de glucógeno (Jung et al., 2007). Además, la **actividad antioxidante** del salvado de arroz sobre la inducción de la liberación de adiponectina y la consiguiente reducción de la resistencia a la insulina se ha probado en líneas celulares de adipocitos 3T3-L1 en las que se observó una reducción del estrés oxidativo (Kim et al., 2011). Por otra parte, **ensayos clínicos** con salvado de arroz han demostrado su capacidad como coadyuvante para controlar los dos tipos de diabetes: diabetes mellitus insulino-dependiente (DM I) y no insulino-dependiente (DM II). Estos ensayos demostraron mejoras en el perfil lipídico, reducción de la glucosa en ayunas y de la hemoglobina glicosilada (Qureshi et al., 2002; Cheng et al., 2010) **(Figura 9)**.

Figura 9: Principales mecanismos de la acción antidiabética.

e) Actividad antineoplásica

La capacidad antioxidante y antiinflamatoria del salvado de arroz también se ha relacionado con su actividad antineoplásica. Las células tumorales dañan la integridad de los tejidos circundantes por medio de la generación de grandes cantidades de RLO, lo que les ayudan a difundirse y generar más daños en el ADN. Asimismo, las células cancerosas también liberan citoquinas que atraen a macrófagos y granulocitos promoviendo la respuesta inflamatoria y la angiogénesis para el transporte de nutrientes y oxígeno hasta el foco canceroso.

Aunque los estudios disponibles que relacionan a los derivados del salvado de arroz con efectos anti-carcinogénicos son muy variados y difieren enormemente en su naturaleza y diseño, en general, apuntan a la actividad **antioxidante**, a la activación de la **respuesta inmunitaria**, a la inhibición de la activación del **NF-κβ** y de la **angiogénesis** como mecanismos responsables de su actividad. Estudios *in vivo* han demostrado los efectos beneficiosos de fitosteril ferulatos en cáncer de colon (Raicht et al., 1980; Deschner et al., 1982; Boateng et al., 2009; Panala et al., 2009; Kim et al., 2012), hígado (Katayama et al., 2003), vejiga urinaria (Kuno et al., 2006), de la cavidad oral (Long et al., 2007), gástrico (Tomita et al., 2008), de mama (Cai et al., 2004) y próstata (Awad et al., 2000; Hudson et al., 2000; Verschoyle et al., 2007). Cabe destacar que se ha reportado una diferente eficacia dependiendo de la pigmentación del arroz, lo cual puede explicarse por la existencia de una relación dosis-dependiente entre la actividad antineoplásica y la concentración de componentes antioxidantes presentes en el salvado de arroz como el γ-tocotrienol y el contenido total de polifenoles (Forster et al, 2013).

4. EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ: ESTUDIOS PREVIOS

Estudios previos con el EESA han demostrado sus excelentes propiedades protectoras en múltiples patologías, sin limitarse al sistema cardiovascular, mediante modelos experimentales *in vitro* e *in vivo*.

En un estudio realizado en líneas celulares de queratinocitos y epidermis reconstruida de humano se demostró que una solución acuosa de EESA poseía una **capacidad antioxidante** comparable a la de la vitamina E, proporcionando **fotoprotección** frente a la exposición a la radiación ultravioleta. Además no se observó ningún indicio de citotoxicidad a las dosis empleadas, lo que unido a la alta compatibilidad con la piel por el carácter hidrófilo del EESA sugiere que el extracto podría emplearse en preparaciones cosméticas para la prevención de daños por exposición a la radiación solar (Santa-María et al., 2010). Esta capacidad antioxidante del EESA también ha sido demostrada en homogeneizados de cerebro de rata. El daño oxidativo sobre las proteínas y lípidos cerebrales se previno gracias a la alta capacidad para la neutralización de radicales libres que presentó el EESA (Parrado et al., 2003).

Adicionalmente, las propiedades **antiproliferativas e inmunomoduladoras** del EESA se han puesto de manifiesto en las líneas celulares MOLT-4 (células T humanas de leucemia linfoblástica aguda) y N13 (línea celular murina de microglía). El EESA indujo una mayor apoptosis en células malignas, sin presentar ningún efecto en las células normales mientras que se incrementó la expresión de TNF-α, mediador de la apoptosis, en las células de microglía (Revilla et al., 2013).

La actividad **hipocolesterolemiante** del EESA ha sido probada en diferentes modelos animales. En ratas Wistar alimentadas con una dieta alta en colesterol o alta en colesterol y ácido cólico suplementada con EESA se observó un incremento del colesterol HDL y una reducción de colesterol total (Revilla et al., 2009). En ratas **Zucker obesas**, la suplementación de la dieta con EESA consiguió paliar múltiples alteraciones asociadas al desarrollo de **síndrome metabólico** a nivel cardiometabólico, vascular e inflamatorio. Se produjeron mejoras en el perfil plasmático (colesterol total, colesterol HDL, triglicéridos, insulinemia, adiponectina, índice aterogénico, HOMA-IR y nitritos) y una disminución de la presión arterial sistólica, de la esteatosis y del desarrollo de resistencia a la insulina (Justo et al., 2013b). Además, se mejoró la función vascular en arterias de conductancia y de resistencia, mediante mejoras en el estrés oxidativo e inflamación en estos vasos (Justo et al., 2013a; Justo et al., 2014). Finalmente, también mejoró la distribución y tamaño de los adipocitos del tejido adiposo epididimal y abdominal, así como las propiedades

proinflamatorias del mismo debidas a la expresión de las citoquinas proinflamatorias IL-1β, IL-6, TNF-α y de la enzima iNOS (Candiracci et al., 2014).

Por último, la suplementación de la dieta de ratones C57BL/6J a los cuales se indujo **obesidad mediante pienso con alta cantidad de grasa**, también consiguió una mejora global del perfil plasmático (colesterol total, triglicéridos, insulina, adiponectina y nitritos), de la inflamación hepática y una atenuación de la resistencia a la insulina, al igual que en ratas Zucker. Asimismo, se produjeron mejoras en la distribución y tamaño de los adipocitos del tejido adiposo epididimal, y una menor expresión de IL-1β, IL-6, TNF-α, PPAR-γ y Emr1y de la infiltración de macrófagos y de su polarización a macrófagos M1 (Justo et al., 2016).

Estos efectos justifican el interés del estudio de las propiedades del EESA en el estado inflamatorio y prooxidante que determina el avance de la enfermedad aterosclerótica.

5. ATEROSCLEROSIS, PROBLEMA DE SALUD PÚBLICA

Las enfermedades cardiovasculares son actualmente la **principal causa de muerte** a nivel mundial. Las cifras publicadas por la Organización Mundial de la Salud (OMS) en septiembre de 2016 estiman que 17.5 millones de personas mueren cada año a causa de enfermedades cardiovasculares, lo que supone un 31% del total de las muertes en todo el mundo (who, 2016). Más del 75% de estas muertes se producen en países con ingresos medios-bajos debido a un menor acceso a tratamientos de urgencia. No obstante, la OMS ha reconocido a la **aterosclerosis como la enfermedad epidémica** más preocupante en el mundo occidental ya que sus manifestaciones más frecuentes, la cardiopatía isquémica y las enfermedades cerebro-vasculares tienen una elevada tasa de morbilidad y causan el 80% de las muertes por enfermedad cardiovascular.

La aterosclerosis es una enfermedad degenerativa de las arterias, en las que se produce un **acumulo de colesterol** y otros tipos celulares en la pared de las arterias causando una **inflamación sistémica** de bajo nivel. Su avance puede precipitarse cuando se dan de forma concomitante otras enfermedades del sistema cardiovascular. Por ello, la prevención primaria de la aterosclerosis pasa por la eliminación de sus factores de riesgo prevenibles, entre los que encontramos la hipertensión arterial, diabetes mellitus, tabaquismo y dislipidemia. Además se recomienda un estilo de vida saludable mediante el ejercicio, el control de peso, y una alimentación sana y balanceada (Piepoli et al., 2016).

En la actualidad existen múltiples herramientas para el cálculo del riesgo cardiovascular. Las más populares son las tablas **Framingham** y **SCORE**, que a partir de variables como la edad, el colesterol total, LDL y HDL, el tabaquismo o la presencia de otras enfermedades como la diabetes o la hipertensión calculan el riesgo de sufrir eventos cardiovasculares (Framingham) o morir a causa de ellos (SCORE) en los próximos 10 años. Estas tablas, en combinación con los niveles de LDL plasmático, son una herramienta clave en la práctica clínica para decidir positivamente sobre la introducción de un tratamiento farmacológico (Piepoli et al., 2016).

Entre los **fármacos** que se emplean actualmente se encuentran inhibidores de la enzima HMG-CoA-Reductasa (estatinas), activadores del receptor PPAR-α (fibratos), secuestrantes de ácidos biliares (resinas de intercambio iónico), inhibidores de la lipolisis y por ende de la secreción de VLDL (niacina), inhibidores selectivos de la absorción de colesterol (ezetimibe) y más recientemente, anticuerpos que intervienen en el reciclaje del receptor de LDL (inhibidores de PCSK9). Estos fármacos van a tratar de reducir los niveles de colesterol por debajo de los límites máximos recomendados (200 mg/del de colesterol total, 100 ml/dl de colesterol LDL y 150 mg/dl de triglicéridos) y aumentar el colesterol HDL por encima de 35 mg/dl en el hombre y 40 mg/dl en la mujer (Piepoli et al., 2016).

6. SÍNTESIS Y METABOLISMO DEL COLESTEROL

El colesterol es una biomolécula fundamental para el funcionamiento del organismo. Es el precursor de la síntesis de hormonas esteroideas y corticoesteroides, de ácidos biliares y de la vitamina D y da fluidez a las membranas celulares. La mayor parte del colesterol plasmático, un 80% aproximadamente, no procede de la dieta, sino de la síntesis endógena, principalmente en el hígado, a partir de su precursor Acetil-CoA. La síntesis y metabolismo del colesterol deben estar estrechamente regulados para prevenir su sobreacumulación, pero garantizando que exista una cantidad suficiente para los procesos metabólicos en los que participa. El paso limitante de la síntesis de colesterol es la enzima HMG-CoA-Reductasa. Los niveles de expresión y actividad de HMG-CoA-Reductasa responden, mediante mecanismos de retroalimentación, a la cantidad de colesterol plasmático. Así, la síntesis del enzima es controlada por el factor de transcripción SREBP (Brown et al., 1997), y su degradación por ubiquitinación y degradación proteolítica (DeBose-Boyd, 2008). Por otra parte, su actividad se regula por inhibición competitiva, a nivel alostérico, mediante fosforilación, y mediante hormonas (Beg et al., 1978).

El colesterol plasmático debe ser transportado en **lipoproteínas** debido a su carácter lipófilo. Las lipoproteínas se componen de colesterol, triglicéridos, fosfolípidos y

unas proteínas anfipáticas denominadas apolipoproteínas (Apo) que median la interacción con diferentes tipos celulares o receptores.

La absorción intestinal de colesterol comienza con la formación de micelas mixtas de colesterol y ácidos grasos libres procedentes de la dieta. Una vez en el enterocito, colesterol y ácidos grasos pueden ser devueltos a la luz intestinal mediante los transportadores ABCG5/8, localizados en la membrana apical, o ser reesterificados y formar parte de los **quilomicrones**. En su transporte hasta el hígado, los quilomicrones ceden triglicéridos al músculo esquelético y tejido adiposo. El colesterol de los quilomicrones remanentes se suman al sintetizado *de novo* en el hígado para formar las **VLDL**. Al igual que los quilomicrones, las VLDL interaccionan con lipasas endoteliales y se van descargando de triglicéridos pasando por unas lipoproteínas de peso intermedio (**IDL**) hasta formar las **LDL**, cuya función es de distribuir el colesterol a los tejidos. Las células captan estas LDL por endocitosis mediada por el receptor de LDL que reconoce ApoB-100 y ApoE **(Figura 10)**.

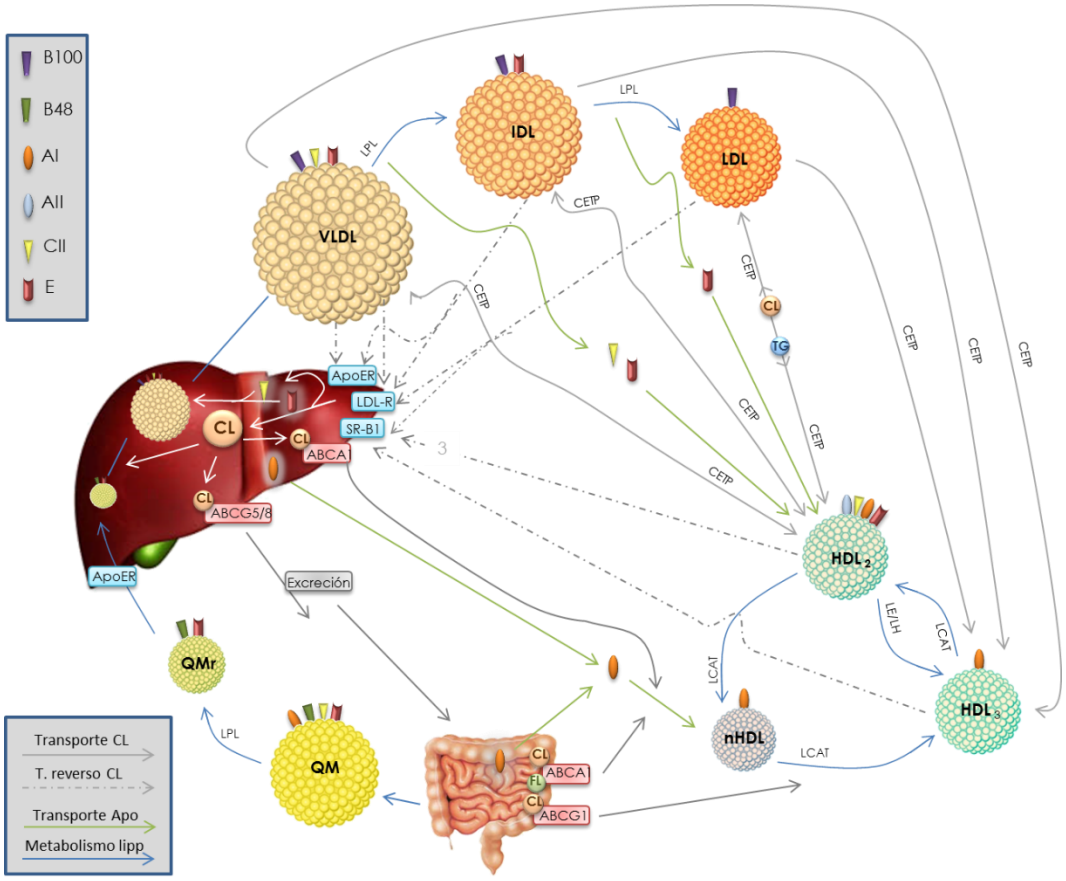

Figura 10: transporte de colesterol en el organismo a través de las lipoproteínas.

El transporte reverso del colesterol desde los tejidos al hígado es mediado por las **HDL**. Una vez en el hígado, el nuevo colesterol hepático será empleado para la síntesis de nuevas VLDL y otra parte será excretada en forma libre con la bilis junto con las sales biliares que también son sintetizadas en el hígado a partir de colesterol (von Eckardstein et al., 2001).

7. FISIOPATOLOGÍA DE LA ATEROSCLEROSIS

La aterosclerosis es un proceso degenerativo vascular, que conduce a la formación de **depósitos de lípidos** en la pared de las arterias, inflamación, estrechamiento de la luz y endurecimiento de los vasos. En la fisiopatología de la enfermedad intervienen numerosos procesos moleculares y celulares que desencadenan una respuesta inflamatoria sistémica, presente en todas las fases de la aterosclerosis.

El **endotelio** es una barrera permeable pero selectiva entre la sangre y los tejidos. El daño en el endotelio se considera el primer paso en el proceso patogénico de la aterosclerosis ya que incrementa su permeabilidad a lipoproteínas y a células del sistema inmune. Este daño puede producirse por diversas causas, como el estrés hemodinámico debido a las turbulencias en el flujo sanguíneo que se producen en las bifurcaciones de las grandes arterias y que se ve acentuado en condiciones de hipertensión arterial, el estrés metabólico derivado de condiciones de hipercolesterolemia, hiperglicemia o hiperhomocisteinemia, o la presencia de infecciones persistentes (citomegalovirus o *Chlamydia pneumoniae*) (Xu et al., 2007).

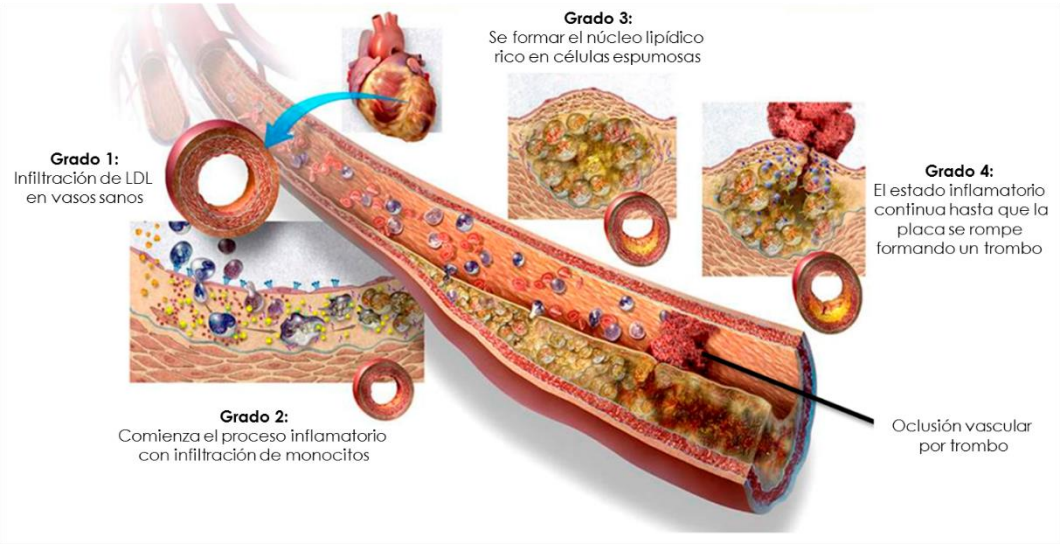

Figura 11: grados del avance de la placa aterosclerótica.

Las partículas de LDL nativas no son aterogénicas *per se*, pues no son reconocidas por los macrófagos. El transporte reverso de colesterol por parte de las HDL es especialmente protector en estos primeros estadios de la enfermedad pues se previene la permanencia prolongada de LDL en la pared vascular. Sin embargo, si las partículas de LDL permanecen el tiempo suficiente en el espacio subendotelial pueden sufrir modificaciones, incluyendo oxidación, lipolisis, proteólisis y agregación su acumulación **(Figura 11, Grado 1)** (Lusis, 2000).

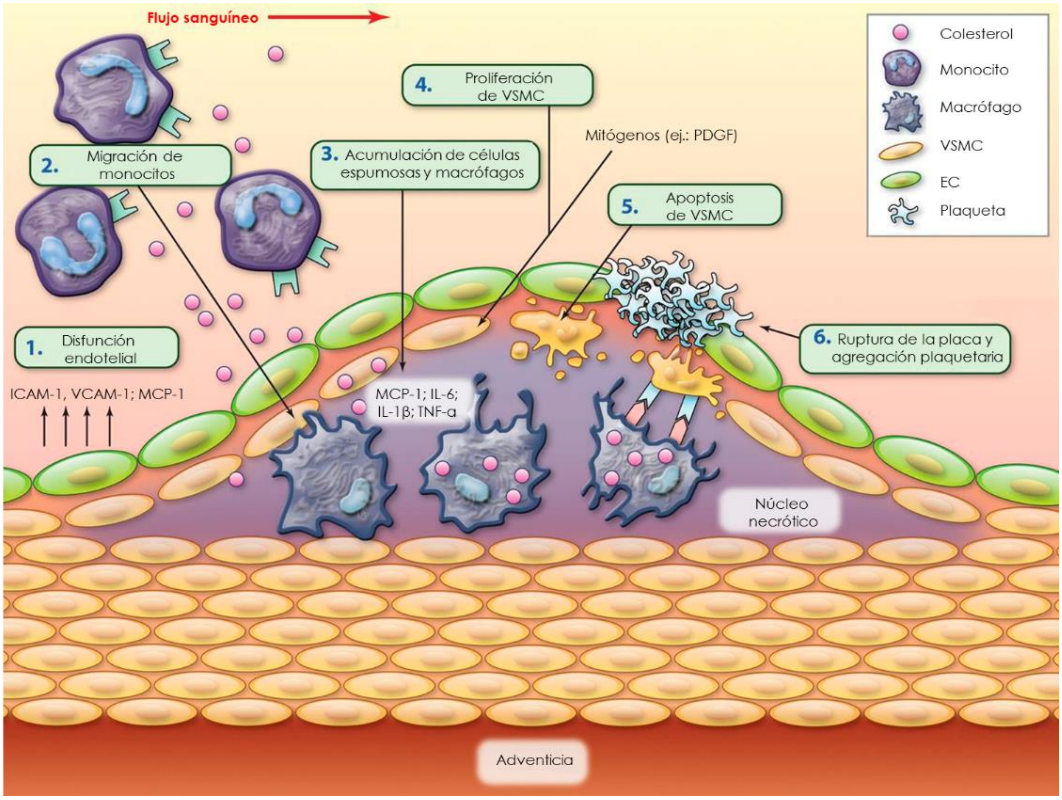

Figura 12: Evolución de la placa aterosclerótica. Traducido de Wang et al., 2012.

Las partículas de LDL oxidadas (**oxLDL**) estimulan a las células endoteliales circundantes a producir citoquinas proinflamarias como TNF-α o IL-1β, **moléculas de adhesión** como E-selectina, P-selectina, VCAM-1 (vascular cell adhesion molecule-1) e ICAM-1 (intracelular cell adhesion molecule-1) (Blankenberg et al., 2003) y factores de crecimiento como M-CSF (macrophage colony-stimulating factor) y pueden ocasionar una reducción en la producción de NO. Al foco dañado acudirán monocitos (que una vez en la pared madurarán a macrófagos), células dendríticas, mastocitos, plaquetas y linfocitos T

y B. Sin embargo, los neutrófilos no participan en el proceso aterosclerótico (Gradinaru et al., 2009) **(Figura 11, Grado 2)**.

Una vez en la pared vascular, los macrófagos fagocitan las LDL modificadas, cargándose de lípidos y transformándose en **células espumosas**, núcleo de la placa de ateroma. Esta captación de lípidos modificados está mediada por receptores de tipo "*scavenger*" localizados en la membrana de los macrófagos, de los cuales SR-A y CD36 son los más relevantes. Las células espumosas pueden lisarse, originando un núcleo necrótico y liberando cristales de colesterol y enzimas catalíticas al espacio intercelular, lo que potenciará el proceso inflamatorio local (Yu et al., 2013). Enzimas como las metaloproteinasas (MMP), que son liberadas al espacio extracelular, degradarán la matriz extracelular, permitiendo el crecimiento de la placa **(Figura 12)**.

A medida que el acumulo de lípidos va aumentando en las células espumosas, lo que originalmente era una estría grasa puede evolucionar a una **placa fibrosa (Figura 11, Grado 3)**. Con objeto de estabilizar la placa, células musculares lisas migran al foco aterosclerótico y se produce un depósito de colesterol. Adicionalmente, se producirá una **hipertrofia** del vaso para asegurar que la luz sea suficiente para garantizar el flujo de sangre a los tejidos. Por el contrario, el crecimiento rápido de núcleo lipídico, la síntesis limitada de colágeno y la degradación de la matriz extracelular generan **placas inestables**. La ruptura de la capa fibrosa y la trombosis y oclusión de vasos secundaria a la ruptura son la causa de la gran mayoría de infartos de miocardio y accidentes cerebrovasculares **(Figura 11, Grado 4)** (Libby, 2002).

8. PATOLOGÍAS Y ALTERACIONES FUNCIONALES ASOCIADAS A LA HIPERCOLESTEROLEMIA Y PROCESO ATEROSCLERÓTICO

A largo plazo, la hipercolesterolemia y el proceso aterosclerótico derivado de ella traen asociado el desarrollo de otras patologías o alteraciones funcionales que pueden potenciar aún más el estado de inflamación y estrés oxidativo sistémico, acelerando el desarrollo de la placa aterosclerótica. Estas alteraciones serán objeto de estudio en esta tesis.

a) Disfunción endotelial y remodelado vascular

La disfunción endotelial es un factor predictivo de aterosclerosis, la cual se inicia con pequeñas lesiones en el endotelio. Además muchos de los factores de riesgo de la aterosclerosis también promueven disfunción endotelial.

La hipercolesterolemia produce la activación de NADPHox y de la producción de superóxido, que reaccionará con el NO, disminuyendo su biodisponibilidad para la relajación vascular. Una elevada cantidad de lípidos plasmáticos también promueven la aparición de dimetilarginina asimétrica (**ADMA**) (Bouras et al., 2013), que es un inhibidor competitivo de la enzima óxido nítrico sintasa endotelial (**eNOS**), la cual puede desacoplarse, incrementando la producción de anión superóxido en detrimento de NO. El **exceso de superóxido** ocasionará a la modificación oxidativa de LDL dando lugar a las oxLDL, que juegan un papel crucial en la disfunción endotelial y en el desarrollo de aterosclerosis, por diferentes mecanismos: (i) la activación de endotelina-1 (Xu et al., 2008), inductor de NADPHox; (ii) activación de metiltransferasas dependientes de S-adenosilmetionina, que incrementan la síntesis de ADMA (Warnholtz et al., 2001); (iii) incremento de la síntesis de caveolina-1, inhibidor de la actividad de eNOS (Davignon et al., 2004) y (iv) por el incremento de la expresión de moléculas de adhesión y otras moléculas quimiotáticas, la promoción de la migración y proliferación de VSMC y macrófagos y otros leucocitos en el foco aterosclerótico, como se ha comentado previamente.

La aterosclerosis es iniciada por pequeños **daños en el endotelio** vascular. En condiciones fisiológicas, las células endoteliales dañadas son reemplazadas por células adyacentes, que proliferan y migran hasta el foco dañado. Sin embargo, cuando la tasa de apoptosis supera la capacidad de reemplazo, el proceso es asistido por células progenitoras endoteliales (**EPC**) residentes en la pared vascular, o circulantes que derivan de la medula ósea o se diferencian a partir de monocitos. Su estudio resulta de interés ya que existe una relación inversa entre los niveles de EPC circulantes y el riesgo de padecer aterosclerosis (Vasa et al., 2001).

Si bien la disfunción endotelial ocurre en las primeras fases del desarrollo de aterosclerosis, el remodelado vascular es indicativo de un estado avanzado. En el proceso aterosclerótico, el crecimiento de la placa supone un **estrechamiento del lumen**. Además se produce un **engrosamiento de la pared** (elevada relación íntima/media) por la proliferación de VSMC. El objetivo del remodelado vascular es mantener el flujo de sangre a los tejidos e incluye una hipertrofia compensatoria del vaso a nivel local (Ward et al., 2000). La hipertrofia adaptativa conseguirá en muchos casos mantener un lumen cercano

a la normalidad. No obstante, supone un mayor riesgo de placa inestable a largo plazo, mientras que a corto plazo el fracaso de esta hipertrofia compensatoria o la ruptura de placas inestables supondrán la oclusión del vaso, con el riesgo de isquemia (Ward et al., 2000).

b) Desarrollo de hígado graso no alcohólico

El hígado graso no alcohólico se da cuando se acumulan **triglicéridos y ácidos grasos** de forma excesiva en el hígado. Aunque los principales factores de riesgo para el desarrollo de hígado graso son la obesidad, la diabetes mellitus tipo II y el síndrome metabólico (Angulo, 2007), un estado de hipercolesterolemia crónica también puede derivar en el acúmulo de colesterol en el hígado a consecuencia de la **lipotoxicidad** ocasionada por un mayor estrés oxidativo, la disfunción mitocondrial y el estado inflamatorio sistémico que conlleva un nivel elevado de colesterol de forma crónica (Kim et al, 2014). Así mismo, el desarrollo de hígado graso supone un factor de riesgo para una mayor incidencia de eventos cardiovasculares (Targhe et al., 2008; Edens et al., 2009). La esteatosis está asociada a una mayor expresión hepática de la citoquinas proinflamatorias, como TNF-α e IL-6, que incrementarán a su vez la producción de proteína C reactiva (CRP). Además, el TNF-α puede interferir con el receptor hepático de la insulina así como en su cascada de señalización hepatocelular, causando resistencia a la insulina a nivel hepático y sistémico (Ryden et al., 2007; Gupta et al., 2007). En condiciones de hígado graso, el estímulo mediado por la insulina para la inhibición de la secreción de partículas de VLDL desde el hígado es menos efectivo, lo cual tiene como consecuencia el incremento plasmático de partículas de LDL y VLDL (Adiels et al., 2006). Además, pacientes afectados de hígado graso no alcohólico producen una mayor secreción hepática de PAI-1 (plasminogen activator inhibitor-1) (Bansilal et al., 2007) y del precursor de angiotensina-II, el angiotensinógeno (Bataller et al., 2003), asociados a con un mayor riesgo de aterosclerosis.

c) Envejecimiento celular y apoptosis

La incidencia de aterosclerosis aumenta con la edad. A su vez, los mecanismos patogénicos implicados en la aterosclerosis promueven el envejecimiento a nivel celular (Uryga et al., 2016). Vasos envejecidos y aquellos en los que un proceso ateroscleróticoestá presente comparten un gran número de características como una disminución del número de VSMC en la lámina media, aumento de la deposición de colágeno y de los estímulos

proinflamatorios, o la fractura de los filamentos de elastina, lo cual puede provocar la dilatación e incrementar el tamaño del lumen (Zieman et al., 2004). El **recambio excesivo** de VSMC y células endoteliales que se produce en la pared vascular durante la aterosclerosis traerá consigo un acortamiento de los **telómeros** y eventualmente un estado de senescencia caracterizado por la incapacidad irreversible de las células para dividirse (Samani et al., 2001). Al igual que el acortamiento de telómeros, los daños acumulados en ADN, fruto del elevado estrés oxidativo presente en la aterosclerosis, derivan en senescencia y apoptosis. Mientras que la apoptosis de VSMC **puede incrementar la inestabilidad de la placa**, ya que estas son las principales productoras de las fibras de colágeno, la apoptosis de macrófagos y células espumosas puede tener **un efecto beneficioso** si los cuerpos apoptóticos son retirados, pudiéndose incluso frenar la acumulación de lípidos en la placa (Kockx et al., 2000).

d) Disfunción mitocondrial

Las mitocondrias son una de las principales fuentes de estrés oxidativo a nivel celular, y a su vez constituyen una diana directa de las ROS recién sintetizadas. Los daños oxidativos ocasionados dan lugar a proteínas disfuncionales, propiciando el desacoplamiento de la respiración oxidativa mitocondrial, aumentando consecuentemente la síntesis de anión superóxido y la probabilidad del desarrollo de la placa aterosclerótica (Chistiakov et al., 2012) y de procesos de apoptosis que favorecen aún más su desarrollo e inestabilidad (Mallat et al., 2000; Geng et al., 2002).

El número de mitocondrias así como su función y estructura son controlados por mecanismos de **biogénesis** y por los procesos implicados en la **dinámica de su ciclo** de vida. Dado que las mitocondrias están sometidas a un fuerte estrés oxidativo derivado de la respiración oxidativa, acumulan un gran número de daños en sus macromoléculas. Los procesos opuestos de **fusión y fisión** permiten la escisión del material dañado y la elongación del orgánulo mediante fusión de otros más pequeños, dando lugar a mitocondrias completamente funcionales. Este proceso dinámico de intercambio de solutos es clave para el reciclaje y buen funcionamiento de las mitocondrias y debe estar estrechamente regulado para evitar la excesiva fragmentación o elongación de las mitocondrias, que perjudicaría a su funcionalidad, derivando en un mayor estrés oxidativo (Liesa et al., 2009).

9. MODELOS EXPERIMENTALES PARA EL ESTUDIO DE ATEROSCLEROSIS

Desde los años 90, los estudios sobre aterosclerosis han ido creciendo exponencialmente. Durante el siglo XX un largo número de estudios clínicos demostró la relación directa entre los niveles de colesterol plasmático elevados y la incidencia de eventos cardiovasculares (The Scandinavian Simvastatin Survival Study Group, 1994; Hebert et al., 1998). Esta evidencia incrementó el interés por el esclarecimiento de las bases moleculares que afectan al desarrollo de la enfermedad aterosclerótica. Los primeros modelos experimentales se basaron en la **inducción de la enfermedad mediante dietas** altas en grasas saturadas y colesterol (dieta tipo Western) o dietas tipo Western enriquecidas en ácido cólico (dieta tipo Paigen). Estas dietas se administraron a especies animales con una mayor propensión natural al desarrollo de la enfermedad, como son el conejo, el hámster, el pollo, el cerdo y el mono.

Sin embargo, los **roedores no son un buen modelo** para la inducción de hiperlipemia mediante la dieta, ya que no son un reflejo inequívoco de la patología en humano, por varios motivos: (i) los modelos actuales inducen una hipercolesterolemia extrema no fisiológica, (ii) los humanos transportan el colesterol principalmente en partículas de LDL mientras que los roedores lo hacen en partículas de HDL **(Figura 13)**, y (iii) no hay una homología genética entre humanos y roedores en genes que son de gran importancia para el estudio del metabolismo lipídico y la aterosclerosis. A modo de ejemplo, la proteína de transferencia de esteres de colesterol CETP no es expresada por los ratones (Stylianou et al., 2012).

A pesar de todo ello, los modelos animales de esta patología tan compleja siguen constituyendo la base de la investigación de estrategias farmacológicas y nutricionales que retrasen o aminoren las consecuencias de la aterosclerosis en diferentes órganos. Además, los **modelos murinos presentan múltiples ventajas** experimentales, como son su bajo coste de mantenimiento, una alta capacidad reproductiva con ciclos gestacionales cortos y gran número de crías, la similitud con los humanos en los procesos biológicos, el alto conocimiento de su genética y la disponibilidad de técnicas optimizadas de cultivo de células embrionarias pluripotenciales que permiten la obtención de ratones knockout mediante procesos de mutación dirigida.

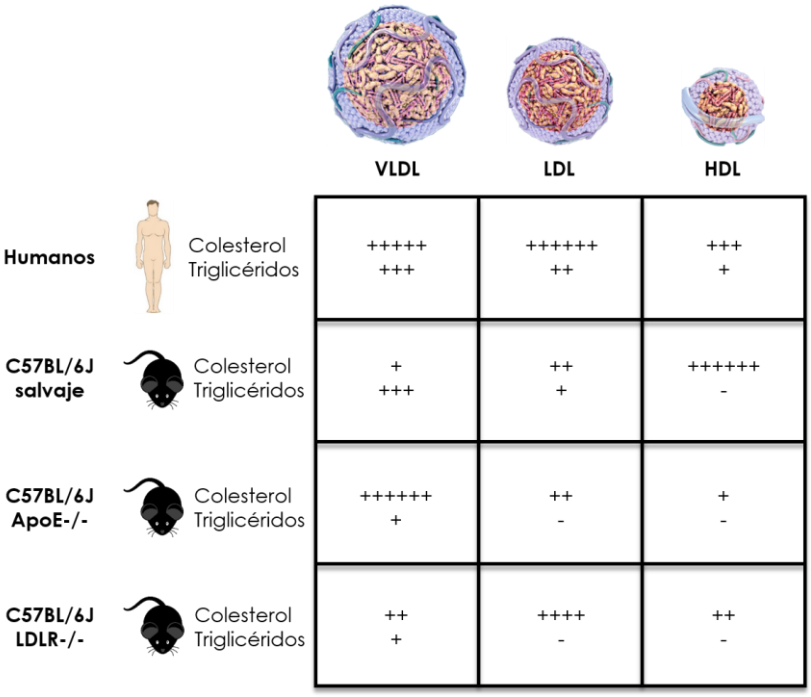

Figura 13: Representación de la cantidad de colesterol y triglicéridos presente en cada tipo de lipoproteína en humanos y ratones de genotipo salvaje, deficientes en apolipoproteína E (ApoE-/-) o en el receptor de las LDL (LDLR-/-). Modificado de Giuseppe Lo Sasso et al., 2016.

Esta última característica supuso un gran avance en los años noventa para el estudio experimental de la aterosclerosis, con el desarrollo de cepas de ratones deficientes en la apolipoproteína E **(ApoE-/-).** El modelo fue desarrollado de forma simultánea e independiente por dos laboratorios en la Universidad de Carolina del Norte y en la Universidad Rockefeller (Nueva York) mediante inactivación genética por recombinación homóloga dirigida (Zang et al., 1992; Plump et al., 1992; Piedrahita et al., 1992). El nuevo modelo genético fue rápidamente aceptado para el estudio de los mecanismos moleculares implicados en el inicio y progreso de la aterosclerosis, y en la enfermedad de Alzheimer por la participación de las ApoE en el transporte de lípidos para el mantenimiento de las membranas neuronales y el aclaramiento del péptido β-amiloide.

Los animales ApoE-/- desarrollan de manera espontánea hipercolesterolemia de alrededor de 500 mg/dL y lesiones ateroscleróticas que son muy similares a los presentes en humanos **(Figura 13)**. Cuando los animales son alimentados con una dieta tipo Western, la hipercolesterolemia se incrementa hasta 3 veces, el desarrollo y extensión de las lesiones se acelera (Meir et al., 2004) y los animales presentan una función endotelial deteriorada

(Meyrelles et al., 2011) **(Figura 14)**. Sin embargo, el hecho de que el transporte de colesterol se realice mayoritariamente en partículas de colesterol VLDL y en quilomicrones, a diferencia de lo que ocurre en humanos, donde el transporte se realiza en partículas de colesterol LDL, llevó al desarrollo de otro modelo genético. El modelo de ratones deficientes en el receptor de las LDL **(LDLR-/-)** fue desarrollado por Joachim Herz y colaboradores en la Universidad de Texas en 1993 (Ishibashi et al., 1993). Estos ratones, a diferencia de los ratones ApoE-/-, transportan el colesterol en partículas LDL, sufren una hipercolesterolemia más moderada y desarrollan placas ateroscleróticas únicamente bajo una dieta tipo Western **(Figuras 13 y 14)**.

Con objeto de obtener modelos más representativos de los procesos patológicos que ocurren en humanos, se han desarrollado cepas de ratones ApoE y LDLR doblemente deficientes, con un perfil plasmático de lipoproteínas similar a los ratones ApoE-/- pero que desarrollan placas ateroscleróticas más rápido y extensas que estos. Del mismo modo, también se han empleado ratones LDLR-/- que sobreexpresan ApoB-100, y desarrollan placas mayores que los ratones ApoE-/- aun teniendo similares niveles de colesterol plasmático. **Otros modelos transgénicos** consisten en la introducción del gen humano de la ApoE o bien de una forma mutada del mismo, como es la ApoE2k o ApoE3-Leiden (Van den Maagdenberg et al., 1993; Hofker et al., 1998), o conejos que sobreexpresan ApoA-I, ApoB-100, lp(a), lecitín-colesterol-aciltransferasa o lipasa hepática humanas (Bosze et al., 2003).

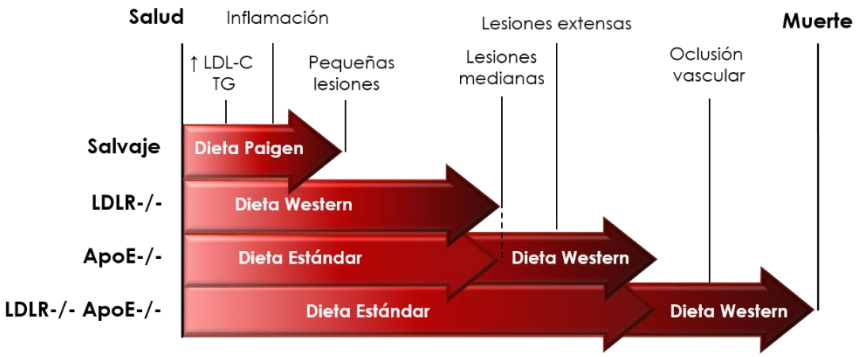

Figura 14: Progresión de las lesiones ateroscleróticas en ratones salvajes, deficientes en apolipoproteína E (ApoE-/-), en el receptor de las LDL (LDLR-/-) o doblemente deficientes ApoE-/- LDLR-/-. Modificado de Ioannis et at., 2012.

JUSTIFICACIÓN Y OBJETIVOS

El salvado de arroz contiene una interesante variedad de compuestos bioactivos entre los que destacan el ácido ferúlico, el γ-oryzanol, tocoferoles y tocotrienoles y una gran variedad de fitosteroles. Estas moléculas han demostrado sus propiedades hipolipemiantes, antiinflamatorias y antioxidantes, y por tanto, resultan de gran interés para la prevención de la aterosclerosis, entre otras enfermedades cardiovasculares. Sin embargo, este potencial está siendo desaprovechado debido a que el salvado de arroz presenta una fuerte tendencia al enranciamiento de sus ácidos grasos, lo cual dificulta y encarece su uso para la industria alimentaria. La extracción enzimática del salvado de arroz da lugar a un producto totalmente soluble en agua debido a la interacción de los componentes oleosos con proteínas de bajo peso molecular, se inactivan las lipasas responsables del enranciamiento y además se consigue un incremento en la concentración de los componentes bioactivos. Estudios previos de nuestro grupo de investigación han determinado el potencial del extracto enzimático de salvado de arroz (EESA) en múltiples marcadores de síndrome metabólico y obesidad. Estos efectos incluyen mejoras en la hipertensión moderada y la resistencia a la insulina, reducción de la disfunción endotelial en arterias de conductancia y resistencia y reducción del estrés oxidativo e inflamación vascular y del tejido adiposo.

Dado el potencial biológico del salvado de arroz para la prevención de enfermedades cardiovasculares, y las mejoras en las características tecnológicas y en la concentración de principios activos introducidas por la extracción enzimática, el objetivo general de esta tesis fue el estudio de los efectos de la suplementación dietética con el EESA sobre progresión de aterosclerosis en un modelo genético con predisposición a la hipercolesterolemia y al desarrollo de placas ateroscleróticas.

Los objetivos específicos planteados para esta tesis fueron los siguientes:

- ✓ Realizar una revisión bibliográfica de los efectos cardiometabólicos y vasculares del salvado de arroz conocidos hasta la fecha, así como de sus productos derivados y principales moléculas bioactivas.
- ✓ Estudiar el efecto del EESA sobre la aterogénesis.
- ✓ Estudiar el mecanismo hipocolesterolemiante del EESA.
- ✓ Estudiar los efectos del consumo del EESA sobre los mecanismos relacionados con el proceso aterosclerótico: el estrés oxidativo y la inflamación.
- ✓ Estudiar los efectos de la suplementación de la dieta con EESA sobre la disfunción endotelial en arterias de conductancia y resistencia.

- ✓ Estudiar los efectos del consumo de EESA sobre la aparición de alteraciones funcionales derivadas del proceso aterosclerótico, como son la esteatosis, la disfunción mitocondrial, la senescencia celular y la apoptosis.
- ✓ Estudiar la biodisponibilidad de una de las principales moléculas bioactivas del EESA, el ácido ferúlico.
- ✓ Identificar las moléculas bioactivas presentes en el EESA que son responsables de los efectos observados.
- ✓ Estudiar los efectos antioxidantes derivados del consumo de ácido ferúlico a medio plazo en células polimorfonucleares humanas.

CAPÍTULO I

La mente que se abre a una nueva idea jamás volverá a su tamaño original

(Albert Einstein, 1879-1955)

CONTRIBUCIÓN DEL ÁCIDO FERÚLICO, DEL γ-ORYZANOL Y DE LOS TOCOTRIENOLES EN LOS EFECTOS CARDIOMETABÓLICOS PROTECTORES DEL SALVADO DE ARROZ

Perez-Ternero C, Alvarez de Sotomayor M, Herrera MD

En revisión en *Journal of Functional Foods*

El salvado de arroz es una excelente fuente de compuestos bioactivos, incluyendo moléculas como el ácido ferúlico, el γ-oryzanol, los fitosteroles y los tocoles. Se conoce que estas moléculas ejercen una protección cardiometabólica, que incluye actividades antidiabéticas y antihipertensivas, siendo la actividad hipocolesterolémica la que destaca de entre las demás. Esta reducción en el colesterol plasmático se ha atribuido fundamentalmente a la reducción de la síntesis hepática y a la inducción de la excreción fecal. Otras actividades de interés de los compuestos bioactivos del salvado de arroz son la actividad antioxidante, debida al incremento de la expresión de enzimas antioxidantes o la reducción de la producción de radicales libres de oxígeno; o las propiedades antiinflamatorias derivadas de la reducción de la activación del NF-κβ y de la producción de citoquinas proinflamatorias. Estas actividades del salvado de arroz y sus moléculas bioactivas contribuyen a la mejora de la disfunción vascular presente en numerosas enfermedades cardiovasculares.

El objetivo de esta revisión es actualizar y reunir los estudios clínicos y preclínicos disponibles hasta la fecha que describen las múltiples actividades biológicas del salvado de arroz y sus principales derivados, responsables de mejoras en la función vascular y endotelial y de numerosos marcadores cardiometabólicos. También se describe la contribución individual de las principales moléculas bioactivas presentes en el salvado de arroz y los problemas tecnológicos que puedan afectar a la actividad biológica de las diferentes presentaciones de los derivados de salvado de arroz disponibles en el mercado.

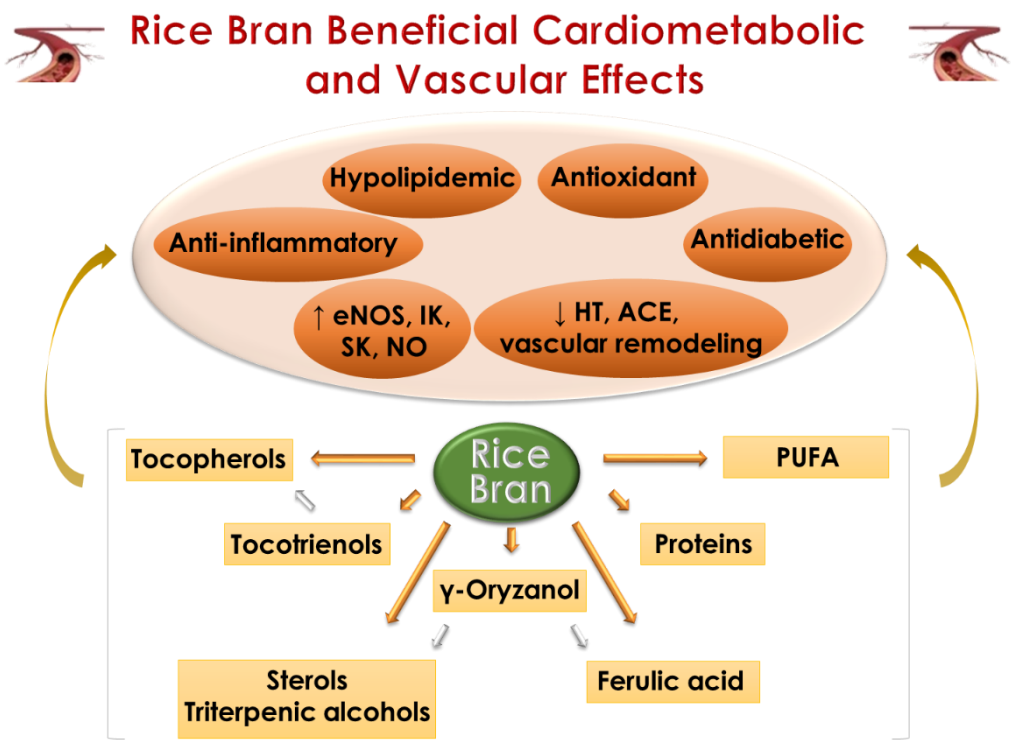

Figura 15: Efectos cardiometabólicos y vasculares beneficiosos del salvado de arroz

CONTRIBUTION OF FERULIC ACID, γ-ORYZANOL AND TOCOTRIENOLS TO THE CARDIOMETABOLIC PROTECTIVE EFFECTS OF RICE BRAN

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Abstract

Rice bran is an excellent nutritional source of bioactive compounds, including phytochemicals such as ferulic acid, γ-oryzanol, phytosterols and tocols. These bioactive molecules have shown cardiometabolic protection, such as anti-diabetic and anti-hypertensive effects, but more importantly, lipid lowering effects due to cholesterol synthesis downregulation and increased faecal excretion. Moreover, rice bran phytochemicals have been described to mitigate oxidative stress by increasing antioxidant enzymes and by reducing oxygen radical production, and to possess anti-inflammatory activity due to downregulation of NF-κβ activation and reduction of pro-inflammatory cytokines production. This review aims to update and summarize clinical and animal studies, describing the multifactorial activities of rice bran and the individual contribution of its main bioactive compounds, namely, γ-oryzanol, ferulic acid, phytosterols, triterpenic alcohols and tocotrienols. Technological factors affecting biological activities of the different rice bran-derived preparations are also discussed.

Keywords: rice bran; cardiometabolic activities; ferulic acid; γ-oryzanol; tocotrienols.

1. Introduction

Cardiovascular diseases are the leading cause of death worldwide, being dietary patterns a major and modifiable exposure risk factor. Nowadays, the Western diet, typical from northern countries of Europe and from the United States, is displacing the cardiovascular-healthy Mediterranean and Asian diets, which are related to a lower incidence of cardiovascular events (Da Silva R, et al., 2009). In contrast, there is an emerging interest in the consumption of foods that are naturally rich in bioactive molecules to treat or prevent cardiovascular diseases, due to its potential to reduce oxidative stress, inflammation, hyperlipidaemia and hyperglycaemia, among other risk factors (Soory, 2012; Riccioni et al., 2012; Chen et al., 2014).

One product that is gathering growing attention is rice bran. Rice bran is produced as a by-product during white rice milling and discarded or allocated to animal feeding. Rice bran is composed of the inner pericarp, testa, aleurone, subaleurone and sometimes the embryo and/or a small part of broken rice. Macro and micronutrient composition of rice bran depends on the soil, the climatologic conditions, the crop and the post-harvesting treatments (Goufo & Trindade, 2015). In general terms, macronutrient composition of rice bran can be summarized as follows: 11-13% protein, 11-12% fibre, 15-20% fat and 40-45% carbohydrates (Moongngarm et al., 2012). But more interestingly, rice bran has been reported to contain high levels of phytochemicals, including γ-oryzanol (a mixture of ferulic acid esters of triterpene alcohols and sterols), tocopherols, tocotrienols (Goufo & Trindade, 2014), carotenoids (Belefant-Miller & Grace, 2010), γ-aminobutyric acid (GABA) (Kim et al., 2015), octacosanol (Chen et al., 2007), phytic acid (Canan et al., 2011), unsaturated fatty acids (with a high ratio of n-6/n-3 polyunsaturated fatty acids) (Orsavova et al., 2015), squalene, phytosterols (Sugihara et al., 2010) and a large number of polyphenols such as flavonoids, anthocyanins, proanthocyanidins and phenolic compounds such as ferulic acid and p-coumaric (Goufo & Trindade, 2014). Rice bran content of its main bioactive molecules is summarize in Table 1.

Epidemiological and experimental studies have reported cardiometabolic protection of rice bran, rice bran oil or different rice bran extracts, alone or in diet supplementation. These biological actions include lipid lowering, antidiabetic, hypotensive, weight loss, antioxidant and anti-inflammatory activities, among others (Sugano & Tsuji, 1997; Cicero & Gaddi, 2001; Islam et al., 2011; Goufo & Trindade, 2014). Most of these activities have been attributed to rice bran unsaponifiable fraction, which includes ferulic acid, γ-oryzanol and tocotrienols. Despite its potential, the use of rice bran for human consumption remains underexploited due to its instability, oily nature and the presence of antinutrients

such as trypsin inhibitors, haemagglutinin-lectin and phytates (Khan et al., 2009). Therefore, an immediate process of stabilization or extraction is required to keep the quality intact and guarantee its safe consumption.

Table 1. Average content (mg/kg) of the main bioactive compounds present in rice bran, rice bran extract and rice bran oil. NA: not available. Data was expressed as mean ± SEM.

	γ-oryzanol	Ferulic acid	Tocotrienols	Reference
Rice bran	2310 ± 40 [a] 1270 ± 80 [b] 7100 ± 0.40 [c]	NA	170 ± 4 [a] 17.1 ± NA [b] 137 ± 15 [c]	[a] Nantiyakul et al., 2012 [b] Wang et al., 2014 [c] Perez-Ternero et al., 2016b
Rice bran infrared heated extract	NA	≈ 0	400.4 ± NA	Kaup et al., 2013
Rice bran enzymatic extracts	3490 ± 100 [a] 8950 ± 0.85 [b]	351 ± 5 [b]	16.52 ± NA [a] 170 ± 15 [b]	[a] Wang et al., 2014 [b] Perez-Ternero et al., 2016b
Rice bran oil (cold press extraction)	17500 ± 180 [b] 9690 ± 130 [c] 6480 ± 370 [d]	0.004 ± 0 [a]	563 ± 4 [c] 620 ± 30 [d]	[a] Siger et al., 2008 [b] Yoshie et al., 2009 [c] Nantiyakul et al., 2012 [d] Pengkumsri et al., 2015
Rice bran oil (hot press extraction)	6230 ± 310	NA	520 ± 30	Pengkumsri et al., 2015
Rice bran oil (hexane)	18490 ± 1520	NA	1110 ± 60	Pengkumsri et al., 2015

Currently, there is a massive body of knowledge supporting the beneficial effects of rice bran-derived products on several cardiovascular diseases. Rice bran contains a mixture of bioactive molecules, which can have interesting synergistic effects. Therefore, the focus nowadays is going towards deciphering the individual contribution of the bioactive molecules and their mechanism of action at the molecular level. Hence, this review aims to update the current research regarding animal and clinical studies of both, rice bran, rice bran extracts and rice bran oil as well as its main isolated bioactive molecules, namely ferulic acid, γ-oryzanol, and tocotrienols, giving light to the mechanism implicated in their activities. For this purpose, a comprehensive search in PubMed and Google Scholar of English language literature was performed using the keywords rice bran, ferulic acid, γ-oryzanol, tocotrienols, sterols and triterpenic alcohols. All original research articles reporting cardiometabolic effects of rice bran, rice bran-derived products, ferulic acid, γ-oryzanol or tocotrienols were included. When the reported source of the isolated molecules was different to rice bran, the article was excluded.

2. Rice bran

The properties of rice bran as a nutraceutical ingredient have long been studied in animal models regarding several cardiovascular diseases, including dyslipidaemia (Kahlon et al., 1990; Hundemer et al., 1991; Newman et al., 1992; Kahlon et al., 1992a; Kahlon et al.,

1992b; Matheson et al., 1995; Kahlon et al., 1996; Ijiri et al., 2015), diabetes (Kim et al., 2010; Chung et al., 2014) and other oxidative stress-related diseases (Chung et al., 2014). The hypolipidemic activity of rice bran was related to increased HDL-C (Kahlon et al., 1992a) and increased bile acid synthesis through induction of CYP7A1 (Matheson et al., 1995) leading to higher cholesterol excretion (Ijiri et al., 2015). Likewise, hyperglycaemia was improved through modulation of enzymes involved in the use and storage of glucose (GK, PEPCK, G6Pase) (Kim et al., 2010; Chung et al., 2014). Table 2 summarizes the animal and clinical studies reporting cardiometabolic protection derived from of rice bran and rice bran-derived products.

However, to achieve these favourable effects, excessive amounts of rice have been often used for experimental diet supplementation, which limit the translational significance of the findings into human nutrition. Nevertheless, clinical studies reported improvement of serum lipids (Kestin et al., 1990; Sanders & Reddy, 1992; Cara et al., 1992; Gerhardt & Gallo, 1998; Rondanelli et al., 2011; Hongu et al., 2014) and hyperglycaemia (Cheng et al., 2010a; Qureshi et al., 2012) using a reasonable dietary supplementation (between 10 and 84 g per day) of rice bran or rice.

Rapid oxidative reactions of the fatty components of rice bran start soon after bran removal and causes rancidity, an undesirable bitter taste and loss of the nutritional and functional value. Moreover, toxic compounds may be produced in the degradation process, which compromises secure consumption (Da Silva et al., 2006). Aiming stabilization, several methods have been used with higher or lower succeed, including ohmic or microwave heating, infrared irradiation, lyophilization, enzymatic extraction, extrusion, dry and moist heating, autoclaving, addition of antioxidants or toasting (Oliveira et al., 2012; Yılmaz et al., 2014; Yılmaz et al., 2015; Goufo & Trindade, 2015). These processes often reduce the amount of phytochemicals but improve its bioavailability (Slavin et al., 2001). The great instability of rice bran due to fast rancidity of the oily components make a call to the need of new products derived from rice bran, such as rice bran extracts (Table 3), rice bran oil (Table 4), or fractions enriched in the active compounds (Tables 5 and 6).

3. Rice bran extracts

Several groups have developed novel techniques to produce rice bran extracts, leading to stabilization of the fatty components due to lipase inactivation and higher yield of the bioactive components (Table 1). Water solubility of the original hydrophobic matter, which is of great interest for the industry, has been also achieved due to peptide interaction with the oily components (Justo et al., 2013a; Justo et al., 2013b; Justo et al., 2014; Candiracci

Table 2. Studies of rice bran-mediated cardiometabolic protective effects.

Pre-clinical

Rice bran

Model	Supplementation	Effect	Reference
Wistar rat	50 g/kg diet	↑ CYP7A1	Matheson et al., 1995
Sprague Dawley rat	181.4 g/kg diet	↓ TG, WAT ↑ Faecal lipids	Ijiri et al., 2015
C57BL/6 mice	70 - 300 g/kg diet	↓ TC, steatosis, glucose, G6Pase, PEPCK, insulin, WAT ↑ SOD, Cat, GPx, GR, PON1, GK, hepatic glycogen	Hundemer et al., 1991 Kim et al., 2010 Chung et al., 2014
Hamster	100 - 600 g/kg diet	↓ TC, LDL-C, TG, steatosis ↑ HDL-C, faecal lipids	Kahlon et al.,1990 Newman et al., 1992 Kahlon et al., 1992a Kahlon et al., 1992b Kahlon et al., 1996

Clinical

Base Disease	Supplementation	Effect	Reference
Mild hypercholesterolemia	Rice bran (11.8 - 84 g)	↓ TC, LDL-C, ApoB ↑ HDL-C/LDL-C, ApoA I/ApoB	Kestin et al., 1990 Gerhardt & Gallo, 1998 Rondanelli et al., 2011
Overweight and obese	Rice bran (15 g) / Rice bran (15 g) + plant sterols (1.8 g)	↓ TC, LDL-C, leptin, F2-isoprostane, BW	Hongu et al., 2014
Diabetes Mellitus type I and II	Rice bran (10 - 20 g)	↓ TC, LDL-C, ApoB, TG, NEFA, glucose, postprandial hyperglycaemia, HbA1c ↑ Adiponectin, insulin	Qureshi et al., 2012 Cheng et al., 2010a
Healthy	Rice bran (10 - 30 g)	↓ TG, postprandial cholesterol chylomicrons	Sanders & Reddy, 1992 Cara et al., 1992

Abbreviations: ApoA: apolipoprotein A; **ApoB**: apolipoprotein B; **BW**: body weight; **Cat**: catalase; **G6Pase**: glucose 6-phosphatase; **GK**: glucokinase; **GPx**: glutathione peroxidase; **GR**: glutathione reductase; **HbA1c**: glycated haemoglobin; **HDL-C**: high-density lipoprotein colesterol; **LDL-C**: low-density lipoprotein cholesterol; **NEFA**: non-esterified fatty acid; **PEPCK**: phosphoenolpyruvate carboxykinase; **PON1**: paraoxonase 1; **SOD**: superoxide dismutase; **TC**: total cholesterol; **TG**: triacylglycerol; **WAT**: white adipose tissue.

et al., 2014; Wang et al., 2014; Justo et al., 2016; Perez-Ternero et al., 2015; Perez-Ternero et al., 2016a; Perez-Ternero et al., 2016b). Although most of the studies used hydrolytic enzymes to perform rice bran extraction, infrared-heated stabilization followed by concentration, and water and ethanol extracts have been used as well. The infrared heated extract achieved improved glucose tolerance (Kaup et al., 2013) and the water extract reduced body weight, lipase activity and plasma triglycerides (Tsutsumi et al., 2000). It is worth to mention that one enzymatic extract showed better lipid lowering properties compared the ethanol extract

(Ardiansyah et al., 2006). This could be explained by a higher extraction of γ-oryzanol or ferulic acid in the enzymatic preparations, but the composition information was available only for the infrared heated extract, which contained almost no ferulic acid but a greater amount of tocotrienols. On the other hand, enzymatic extracts showed wider effects, such as improvement of dyslipidaemia and atherosclerosis (in relation with HMG-CoA reductase inhibition and increased cholesterol excretion) (Wang et al., 2014), reduction of hypertension due to ACE inhibition (Ardiansyah et al., 2006; Ardiansyah et al., 2007) and hyperglycaemia reduction as well as a general improvement on obesity and on the pro-inflammatory and pro-oxidant status present in the Zucker rat genetic model of metabolic syndrome (Justo et al., 2013a; Justo et al., 2013b; Justo et al., 2014; Candiracci et al., 2014) or the diet induced obesity model (Justo et al., 2016) (Table 3). In these experimental models, vascular function resulted directly benefited through upregulation of eNOS and potassium channels protein expression and activity and indirectly through improvement of the above-described parameters.

Despite the interest of rice bran extracts, only one clinical study has been performed with a rice bran extract rich in acylated steryl glucosides, which has found reduced LDL-C, abdominal circumference and subcutaneous fat area compared to the placebo group (Ito et al., 2015).

Hypolipidemic activities of rice bran extracts have been attributed to the presence of γ-oryzanol and phytosterols in rice bran extracts (Ardiansyah et al., 2006; Wang et al., 2014). In contrast, ferulic acid was identified as the main responsible for antioxidant activities, and for the control of glucose metabolism (Kaup et al., 2013; Ardiansyah et al., 2006), despite being rice bran extract more effective than isolated ferulic acid in lowering blood pressure and improving glucose tolerance (Ardiansyah et al., 2007) .

4. Rice bran oil

Rice bran contains 17 % of oil that can be extracted mechanically or chemically for human consumption or for other purposes, mainly in cosmetics or as biodiesel (Lin et al., 2009). Rice bran oil (RBO) has been typically used as an alternate bakery shortening, as a spreadable margarine, as regular oil for cooking or crude as seasoning (Adhikari et al., 2010; Kaur et al., 2012). The method of oil extraction from rice bran has a huge impact on the phytochemical concentration and antioxidant properties of the final product. In a study performed by Pengkumsri N *et al.* (2015), RBO was extracted from black, red and brown rice by hexane, hot pressed, cold pressed, or supercritical fluid extraction methods, and tocols and γ-oryzanol content were measured, being the hexane-extracted oil the one with higher

Table 3. Studies of rice bran extract-mediated cardiometabolic protective effects.

Pre-clinical

Model	Supplementation	Effect	Reference
Non-enzymatic extracts			
Wistar rat	0.01 g infrared heated extract/kg BW	↑ Insulin secretion	Kaup et al., 2013
Sprague-Dawley rat	100 g water extract/kg diet	↓ TG, lipase activity, BW, WAT	Tsutsumi et al., 2000
SHRSP rats	60 g ethanol extract/kg diet	↓ TG, glucose, HT, ACE, $NO_{(x)}$	Ardiansyah et al., 2006
Enzymatic extracts			
Wistar rat	0.75 g/kg BW	↓ TC, LDL-C, Lp(a), TG, steatosis, HMG-CoA, BW, WAT, $NO_{(x)}$ ↑ HDL-C, SOD, TAOC, faecal lipids	Wang et al., 2014
SHRSP rats	60 g/kg diet	↓ TC, LDL-C, TG, glucose, HT, ACE, Nox-1	Ardiansyah et al., 2006 Ardiansyah et al., 2007
Zucker rat	10 - 50 g/kg diet	↓ TC, TG, HT, HOMA-IR, TNF-α,IL-6, IL-1β, iNOS, $O_2^{\cdot-}$, Nox-1, $p22^{phox}$, $NO_{(x)}$ ↑ HDL-C, NO, eNOS, IK_{Ca}, SK_{Ca}, adiponectin, vascular function	Justo et al., 2013a Justo et al., 2013b Justo et al., 2014 Candiracci et al., 2014
C57BL/6 mice	10 - 50 g/kg diet	↓TC, TG, glucose, IR, adiponectin, $NO_{(x)}$, IL-6, IL-1β, TNF-α, EMR1, adipocyte size, WAT ↑ PPAR-γ	Justo et al., 2016
C57BL/6 ApoE-/- mice	10 - 50 g/kg diet	↓ TC, TG, $eNOS^{Thr495}$, Nox-1, $p22^{phox}$, $O_2^{\cdot-}$, endothelial apoptosis, NF-κβ, TNF-α, COX-2, iNOS, macrophage infiltration, oxLDL ↑ CuZn-SOD, atherosclerotic plaque, vascular function	Perez-Ternero et al., 2015 Perez-Ternero et al., 2016a Perez-Ternero et al., 2016b
Base Disease	**Supplementation**	**Effect**	**Reference**
Obese with high LDL-C	Rice bran extract rich in acylated steryl glucosides (30 - 50 mg)	↓ LDL-C, LDL-C/HDL-C, abdominal circumference and subcutaneous fat area	Ito et al., 2015

Abbreviations: ACE: angiotensin-converting enzyme; **BW**: body weight; **EMR1**: EGF-like module-containing mucin-like hormone receptor-like 1; **eNOS**: endothelial nitric oxide synthase; **FAS**: fatty acid synthase; **HDL-C**: high-density lipoprotein cholesterol; **HMG-CoA**: 3-hydroxy-3-methylglutaryl-coenzyme A; **HOMA-IR**: homeostatic model assessment of insulin resistance; **HT**: hypertension; **IK_{Ca}**: intermediate-conductance calcium-activated potassium channel; **IL-1β**: interleukin 1 beta; **IL-6**: interleukin 6; **iNOS**: inducible nitric oxide synthase; **IR**: insulin resistance; **LDL-C**: low-density lipoprotein cholesterol; **LP(a)**: lipoprotein(a); **NO**: nitric oxide; **$NO_{(x)}$**: nitric oxide metabolites; **Nox-1**: NADPH oxidase 1; **$O_2^{\cdot-}$**: superoxide anion; **$p22^{phox}$**: human neutrophil cytochrome b light chain (CYBA); **PPAR**: peroxisome proliferator-activated receptor; **SK_{Ca}**: small-conductance calcium-activated potassium channel; **SOD**: superoxide dismutase; **TAOC**: total antioxidant capacity; **TC**: total cholesterol; **TG**: triacylglycerol; **TNF-α**: tumour necrosis factor alpha; **WAT**: white adipose tissue.

content in these active molecules and with better antioxidant properties (Table 1) (Pengkumsri et al., 2015). However, incomplete elimination of hexane is a limitation that reduces its biological potential. The impact of temperature in the extraction process regarding quality and quantity of the bioactive molecules was analysed as well. While high temperature favours extraction in terms of quantity, it also impairs the antioxidant capacity. In contrast, temperature-controlled processes preserve antioxidant activity to the detriment of the yield (Table 1) (Pengkumsri et al., 2015). The same applies to oil refining processes, where γ-oryzanol is reduced dramatically, reaching only 2% of the initial content (Pestana et al., 2008).

Ferulic acid is not routinely measure in RBO and when it has been measured, the concentration was very low due to the hydrophilic condition of ferulic acid. However, ferulic acid can still be released from γ-oryzanol *in vivo* and be absorbed to perform its biological activities. The extraction process of crude oil reduces the retention of tocopherols, tocotrienols and γ-oryzanol in RBO compared to the oily bodies in raw rice bran. However, there is a net enrichment of these phytochemicals in RBO, especially of tocotrienols (3.31 fold increase) and γ-oryzanol (2.25 fold increase) (Table 1) (Nantiyakul et al., 2012). Several studies have been conducted with full fat rice bran, defatted rice bran, RBO or rice bran unsaponifiable matter to determine which component of rice bran is primarily responsible for the hypolipidemic activities (Kahlon et al., 1990; Newman et al., 1992; Kahlon et al., 1992a; Kahlon et al., 1992b; Kahlon et al., 1996; et al., Wilson et al., 2007). In contrast to defatted rice bran, full fat rice bran, RBO and the unsaponifiable fraction of rice bran improved serum lipid patterns. This data suggests that the cholesterol-lowering activity of RBO relies on the unsaponifiable fraction, represented by γ-oryzanol, despite losses on the activity of the active molecules during the extraction process of RBO, as previously discussed (Kahlon et al., 1992b; Nantiyakul et al., 2012).

RBO contains around 5% of unsaponifiable matter, which greatly accounts for the RBO biological activities (Afinisha Deepam et al., 2012). Table 4 summarizes the animal and clinical studies of rice bran oil-derived cardiometabolic protective effects. The typical composition of crude RBO is 14.1-28 % saturated fatty acids, 32.4-43.7 % monounsaturated fatty acids and 28.2-55 % polyunsaturated fatty acids (Gopala Krishna et al., 2006). More interestingly, RBO contains a number of bioactive compounds in a considerable concentration, which makes RBO the most used rice bran-derived product, with special popularity in eastern countries and North America. Regarding nutritional interventions, it is important to consider whether RBO is added to the diet or replaces saturated or hydrogenated fats, to keep the macronutrients balance. RBO stands out by its hypolipidemic activity due to a high content in γ-oryzanol (Wilson et al., 2000). In this sense,

Table 4. Studies of rice bran oil-mediated cardiometabolic protective effects.

Pre-clinical

Model	Supplementation	Effect	Reference
Wistar rat	50 - 200 g/kg diet	↓ TC, LDL-C, VLDL-C, TG, LPO, steatosis ↑ HDL-C, LDL-R, CYP7A1, SREBP-2, faecal cholesterol, bile acids, SOD, Cat, GR, GPx	Purushothama et al., 1995 Reena & Lokesh, 2007 Chopra & Sambajah, 2009 Reena et al., 2011 Umesha & Naidu, 2012 Sengupta et al., 2014 Tong et al., 2014 Chandrashekar et al., 2014 Umesha & Naidu, 2015
Weaning Wistar rat	100 g/kg diet	↓ CRP, IL-1β, NF-κβ, cPLA2 ↑ SOD, Cat, GPx, GST, adiponectin-R 1/2, PPAR-γ	Rao et al., 2016
Wistar (STZ) rat	100 - 150 g/kg diet	↓ LDL-C, TG, NEFA, steatosis, IR ↑ HDL-C, LDL-R, faecal cholesterol, HMG-CoA, CYP7A1	Chen & Cheng, 2006 Chou et al., 2009
Wistar/NIN rat	100 g/kg diet	↓ TC, LDL-C, TG, steatosis ↑ HDL-C, faecal cholesterol	Sunitha et al., 1997
Sprague-Dawley rat	100 mg/kg BW or 100 - 150 g/kg diet	↓ TC, LDL-C, ApoB, TG, NEFA, HMG-CoA, LCAT, FAS, G6PDH, IDH, ACC, steatosis, TBARS ↑ HDL-C, ApoA I, ABCA1, PPAR-α, PON1	Koba et al., 2000 Ha et al., 2005 Chithra et al., 2015
Sprague-Dawley (STZ) rat	50 - 150 g/kg diet	↓ LPO, 8-hydroxy-2'-deoxyguanosine ↑ SOD, Cat, GPx, coenzyme Q 10, ORAC	Hsieh et al., 2005 Posuwan et al., 2013
ExHC rat	100 g/kg diet	↓ TC, steatosis ↑ HDL-C, faecal cholesterol	Nagao et al., 2001
Albino rats	100 g/kg diet	↓ TC, LDL-C, VLDL-C, steatosis ↑ HDL-C, faecal cholesterol	Sharma & Rukmini., 1986
Albino rats (NDEA)	200 g/kg diet	↓ LPO, AST, ALT, ALP ↑ SOD, Cat, GPx	Rana et al., 2004
Charles Foster rat	200 g/kg diet	↓ TC, TG, BW	Dhara et al., 2012
Hamster	50 - 100 g/kg diet	↓ TC, LDL-C, TG, LPO, cholesterol absorption, HMG-CoA, fatty streak formation ↑ HDL-C, intestinal HMG-CoA	Kahlon et al., 1992a Kahlon et al., 1992b Ausman et al., 2005 Wilson et al., 2007
Chicken	50 - 200 g/kg diet	↓ TC	Kang & Kim, 2016
Mare	240 g	↓ VLDL-C, TG, NEFA ↑ HDL-C, TC	Frank et al., 2005
Monkey	5 - 35% kcal	↓ TC, LDL-C, ApoB	Nicolosi et al., 1991 Wilson et al., 2000

Clinical

Base Disease	Supplementation	Effect	Reference
Hypercholesterolemia	20% kcal	↓ TC, LDL-C	Utarwuthipong et al., 2009
Mild hypercholesterolemia	20 - 50 g or 20% kcal	↓ TC, LDL-C, LDL-C/HDL-C, VLDL-C	Eady et al., 2011 Lichtenstein et al., 1994 Berger et al., 2005
Hyperlipidemia	30 g or cooking oil	↓ TC, LDL-C, oxLDL, TG, CRP, BW	Raguram et al., 1989 Kuriyan et al., 2005 Malve et al., 2010 Zavoshy et al., 2012 Upadya et al., 2015
Hypertension	Cooking oil	↓ TC, LDL-C, TG, HT	Devarajan et al., 2016a
Diabetes Mellitus type II	18 - 30 g or cooking oil	↓ TC, LDL-C, TG, glucose, postprandial hyperglycaemia, HbA1c ↑ HDL-C	Lai et al., 2012 Salar et al., 2016 Devarajan et al., 2016b
Healthy	29 – 75 g or 1/3 dietary fat	↓ TC, LDL-C, VLDL-C, ApoB, TG, LPO	Suzuki & Oshima, 1970a Suzuki & Oshima, 1970b Rajnarayana et al., 2001 Most et al., 2005

Abbreviations: ABCA1: ATP-binding cassette transporter; **ACC**: acetyl-CoA carboxylase; **ALP**: alkaline phosphatase; **ALT**: alanine aminotransferase; **ApoA**: apolipoprotein A; **ApoB**: apolipoprotein B; **AST**: aspartate aminotransferase; **BW**: body weight; **Cat**: catalase; **cPLA2**: phospholipase A2; **CRP**: C-reactive protein; **CYP7A1**: cholesterol 7 alpha-hydroxylase; **FAS**: fatty acid synthase; **G6PDH**: glucose 6-phosphatase; **GPx**: glutathione peroxidase; **GR**: glutathione reductase; **GST**: glutathione S-transferase; **HbA1c**: glycated haemoglobin; **HDL-C**: high-density lipoprotein cholesterol; **HMG-CoA**: 3-hydroxy-3-methylglutaryl-coenzyme A; **IDH**: isocitrate dehydrogenase; **IL-1β**: interleukin 1 beta; **IR**: insulin resistance; **LCAT**: lecithin–cholesterol acyltransferase; **LDL-C**: low-density lipoprotein cholesterol; **LDL-R**: Low-density lipoprotein receptor; **LPO**: lipid hydroperoxide; **NEFA**: non-esterified fatty acid; **NF-κβ**: nuclear factor kappa beta; **ORAC**: oxygen radical absorbance capacity; **oxLDL**: oxidized low-density 3lipoprotein; **PON1**: paraoxonase 1; **PPAR**: peroxisome proliferator-activated receptor; **SOD**: superoxide dismutase; **SREBP**: sterol regulatory element-binding protein; **TBARS**: thiobarbituric acid reactive substances; **TC**: total cholesterol; **TG**: triacylglycerol; **VLDL-C**: very low-density lipoprotein cholesterol.

most of the human and animal studies reported improvement in the lipid profile due to faecal cholesterol and bile acid excretion and regulation of cholesterol synthesis through HMG-CoA reductase, LDL receptor, CYP7A1, SREBP-2, LCAT, FAS and ABCA1 (Table 4).

Additionally, several studies have shown that regular consumption of RBO reduces postprandial and fasting glucose and glycated haemoglobin in streptozotocin-treated rats (Chen & Cheng, 2006; Chou et al., 2009) and humans suffering from diabetes mellitus II (Lai et al., 2012; Salar et al., 2016; Devarajan et al., 2016b). Other activities exerted by RBO in animal models include oxidative stress protection by an increase in antioxidant enzymes as

a result of a high ferulic acid from γ-oryzanol and vitamin E concentration in RBO (Purushothama et al., 1995; Reena & Lokesh, 2007; Chopra & Sambaiah, 2009; Reena et al., 2011; Umesha & Naidu, 2012; Sengupta et al., 2014; Tong et al., 2014; Chandrashekar et al., 2014; Umesha & Naidu, 2015; Rao et al., 2016; Hsieh et al., 2005; Posuwan et al., 2013; Rana et al., 2004) and anti-inflammatory activities via NF-κβ inhibition (Rao et al., 2016).

5. Activities of the main rice bran phytochemicals

5.1 γ-oryzanol, ferulic acid, triterpenic alcohols and sterols

γ-oryzanol is a mixture of steryl and triterpenyl esters of ferulic acid, being cycloartenyl ferulate and 24-methylenecycloartanyl ferulate the major components in the γ-oryzanol from rice (Miller & Engel, 2006). Although γ-oryzanol absorption has been object of several studies, recent works pose the hypothesis that γ-oryzanol is hydrolysed in the gut liberating ferulic acid and triterpenic alcohols or sterols as free molecules, which could be absorbed and have systemic effects (Hallikainen et al., 2014; Bhaskaragoud et al., 2016) (Figure 1). Ferulic acid (3-methoxy-4-hydroxycinnamic acid) is the main phenolic acid occurring in cell walls of seeds and leaves of monocotyledons both free and conjugated to polysaccharides, glycoproteins, polyamines and fatty acids. Several clinical studies try to address the effects of ferulic acid and γ-oryzanol by means of ferulic acid- or γ-oryzanol-rich foods. Only two studies with isolated γ-oryzanol and two more with sterols from rice bran are available. Therefore, more studies are needed using the isolated molecules to yield structure-specific effects in humans without interaction of other components present in these ingredients. Table 5 summarizes the animal and clinical studies of ferulic acid-, sterols-, triterpenic alcohols- and γ-oryzanol-derived cardiometabolic protective effects.

γ-oryzanol exerts better lipid-lowering performance than ferulic acid alone due to a double mechanism taking action in the gut and in the liver. The sterol moiety acts increasing cholesterol excretion due to physicochemical interference with micellar solubilisation of cholesterol in the gut lumen and upregulates the basolateral sterol exporter ATP-binding cassette (ABC)-A (Brauner et al., 2012). After absorption, the ferulic acid moiety inhibits HMG-CoA reductase-derived synthesis of cholesterol in the liver (Wang et al., 2015). In this sense, many studies have reported improvement in the lipid profile after ferulic acid or γ-oryzanol consumption by means of total cholesterol, LDL-C, NEFA and triacylglycerol reduction and increase HDL-C in different animal models (Table 5). This improvement in blood lipids achieved plaque development prevention in the ApoE-/- model of atherosclerosis (Kwon et al., 2010). In addition, two clinical studies with γ-oryzanol are available reporting lipid profile improvement in humans (Ishihara et al., 1984; Sasaki et al., 1990).

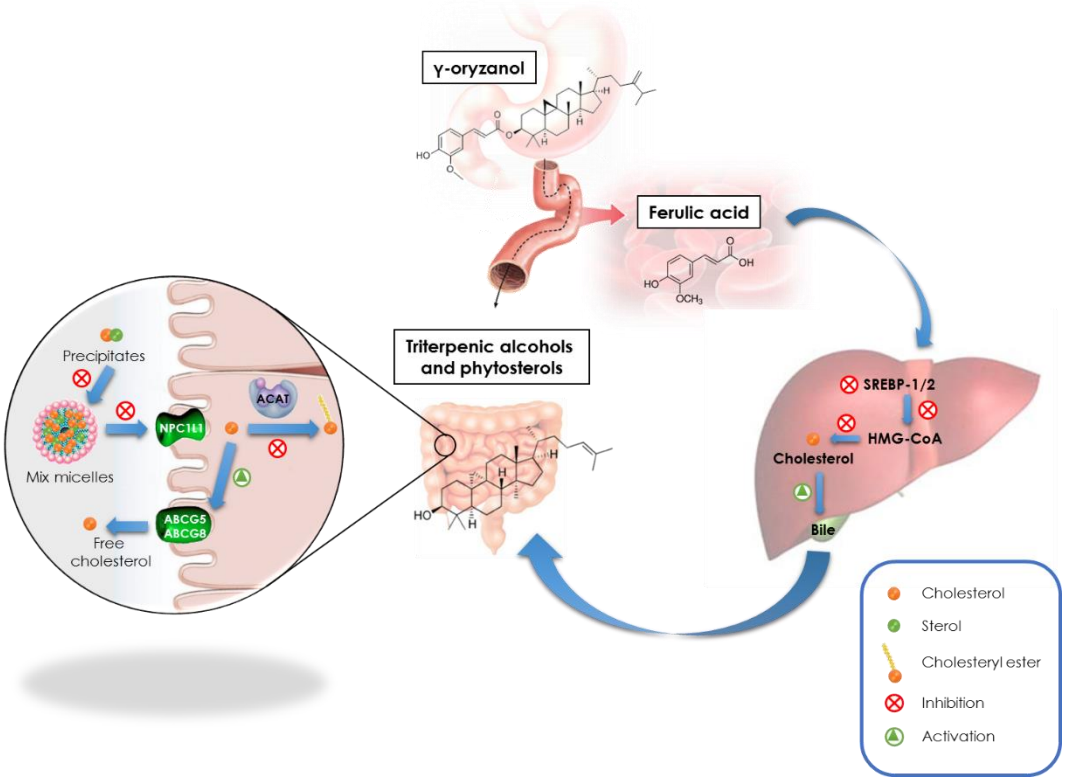

Figure 1: γ-oryzanol dual lipid lowering activities. γ-oryzanol is hydrolysed in the intestine by pancreatic lipases, releasing sterols and ferulic acid, which will show different lipid lowering mechanisms. Sterols are not absorbed and remain in the intestine, and interact with dietary cholesterol, leading to its precipitation. Moreover, they displace cholesterol from the mixed micelles and once in the enterocyte, prevent cholesterol reesterification by inhibition of Acetyl-Coenzyme A acetyltransferase (ACAT). Non-esterified cholesterol returns to gut lumen by ABCG5/8 (ATP-binding cassette) transporters, which are upregulated by sterols. All these mechanisms contribute to higher cholesterol excretion. On the other hand, ferulic acid is absorbed and reaches the liver, where it reduces SREBP-1/2 (Sterol regulatory element-binding protein) expression, which is necessary for HMG-CoA (β-hidroxi-β-metilglutaril-coenzima A) reductase activation, reducing *de novo* synthesis of cholesterol. γ-oryzanol also increases bile flow and total bile acid output, closing the cycle.

Like other phenols, ferulic acid and γ-oryzanol as a source of ferulic acid, exert antioxidant activity in animal models through donation of one hydrogen atom from the phenolic hydroxyl group, upregulation of antioxidant enzymes and downregulation of pro-oxidant enzymes such as NADPHox (Sri Balasubashini et al., 2003; Roy et al., 2013; Alam et al., 2013; Senaphan et al., 2015; Song et al., 2014). Ferulic acid reduction of oxidative stress cell injury, as that produced by streptozotocin in the pancreas to experimentally induce diabetes mellitus II, has shown that lower levels of free radicals facilitates the proliferation of β-cells, which after secreting insulin lead to reduction of glucose and HbA1c. Other ferulic acid-mediated effects showing improved glucose metabolism in animal models of diabetes and obesity include reduction of HOMA-IR (Wang et al., 2015), G6Pase (Naowaboot et al., 2016) and increased insulin and GK (Jung et al., 2007).

Table 5. Studies of ferulic acid-, γ-oryzanol-, triterpenic alcohols and sterols-mediated cardiometabolic protective effects.

Pre-clinical

Model	Supplementation	Effect	Reference
Ferulic acid			
Wistar (STZ) rat	10 – 50 mg/kg BW	↓ TC, TG, NEFA, glucose, TBARS, IL-1β, TGF-β ↑ Cat, SOD, GR	Sri Balasubashini et al., 2003 Roy et al., 2013
Wistar (CCl4) rat	20 mg/kg BW	↓ TC, TG, NEFA, steatosis	Marimuthu et al., 2013
Wistar (L-NAME) and SHR rat	50 mg/kg BW	↓ LPO, HT, left ventricular remodelling, AST, ALT, ALP ↑ SOD, Cat, vasorelaxation, NO	Alam et al. 2013
Sprague-Dawley rat	30 - 60 mg/kg BW or 0.5 g/kg diet	↓TC, LDL-C, TG, LPO, NEFA, steatosis, HMG-CoA, FAS, SREBP-2, glucose, HOMA-IR, HT, vascular remodelling, $p47^{phox}$, TNF-α, AST, ALT, ALP, BW, ↑ HDL-C, NO, eNOS	Senaphan et al., 2015 Wang et al., 2015
Sprague–Dawley (STZ) rat	30 - 60 mg/kg BW	↓ TC, TG, glucose, CK, LDH, $NO_{(x)}$, TBARS, ALT, AST ↑ HDL-C, SOD, GSH, GST, HO-1	Song et al., 2014
SHR rats	1 to 100 mg/kg BW	↓ HT	Suzuki et al., 2002
SHRSP rats	9.5 - 10 mg/kg BW	↓ TC, LDL-C, TG, glucose, ACE, HT, Nox-1	Ardiansyah et al., 2006 Ardiansyah et al., 2007 Ardiansyah et al., 2008
C57BL/6J	5 g/kg diet	↓ TC, TG, steatosis, FAS, TBARS, FAS, BW ↑ HDL-C, Cat, GPx, PON1	Ohara et al. 2009 Jin Son et al., 2010
C57BL/KsJ db/db mice	0.05 g/kg BW	↓TC, LDL-C, glucose ↑ Insulin, hepatic glycogen synthesis, GK	Jung et al., 2007
ICR mice	25 - 50 mg/kg BW	↓ TC, TG, NEFA, steatosis, SREBP-1, FAS, glucose, ACC, PEPCK, G6Pase, IR, leptin ↑ Adiponectin, CPT1A, PPAR-α	Naowaboot et al., 2016
ApoE-/- mice	0.2 g/kg diet	↓ TC, ApoB, ApoB/ApoA-I, ACAT, atherosclerotic plaque, TBARS, WAT ↑ SOD, Cat, GPx, GR, PON1	Kwon et al., 2010
Hamster	5 mg/kg diet	↓TC, LPO, HDL-C	Wilson et al., 2007
Rabbit	0.2 g/day	↓ TG, atherosclerotic plaque	Wang et al., 2004
Rat aorta	0.00001 - 30 mM	Endothelium dependent and independent vasorelaxation ↑ ACh response ↓O_{2^-}	Suzuki et al., 2007 Chen et al., 2009 Fukuda et al., 2015
Oryzanol			
Wistar rat	5 - 20 g/kg diet	↓ TC, LDL-C, VLDL-C, steatosis ↑ HDL-C, faecal cholesterol, bile flow and total bile acid output	Shinomiya et al., 1983 Seetharamaiah & Chandrasekhara, 1990

Wistar (STZ) rat	50 - 100 mg/kg BW or 52.5 g/kg diet	↓ LPO, glucose, IR ↑ SOD, GSH, lipid metabolism	Cheng et al., 2010b Ghatak & Panchal, 2012a
Wistar (Triton WR-1339) rat	50 - 100 mg/kg BW	↓ TC, LDL-C, VLDL-C, TG, LPO, LDL-C/HDL-C ↑ HDL-C, GSH	Ghatak & Panchal., 2012b
Sprague-Dawley rat	0.1 - 10 g/kg BW	↓ TC, LDL-C, TC/HDL-C, TG, NEFA, steatosis, FAS, SREBP-2, HMG-CoA, LDH, glucose, IR, CRP, IL-6, ALT, AST, BW ↑ HDL-C, adiponectin	Nakayama et al., 1987 Sakamoto et al., 1987 Wang et al., 2015
C57BL/6 mice	0.085 – 15 mg or 5 g/kg diet	↓ TC, TG, steatosis, FAS, G6PDH, TBARS, NF-κβ, BW ↑ HDL-C, SOD, Cat, GPx, GR, PON1, faecal cholesterol, adiponectin	Ohara et al., 2009 Jin Son et al., 2010 Nagasaka et al., 2011
Hamster	5 - 10 g/kg diet	↓TC, IDL-C+LDL-C, VLDL-C, TG, LPO, cholesterol absorption, atherosclerotic plaque ↑ HDL-C, faecal cholesterol	Rong et al., 1997 Wilson et al., 2007
Rabbit	10 g/kg diet	↓ Cholesterol uptake by macrophages, oxLDL	Hiramatsu et al., 1990

Triterpene alcohols and sterols

C57BL/6 mice	0.5 -12.5 µg triterpene alcohols or sterols/g BW	↓ TC, GIP, postprandial hyperglycaemia, steatosis, leptin, SREBP-1, FAS, BW, WAT	Fukuoka et al., 2014 Okahara et al., 2016
Hamster	2.4 - 4.8 g sterols/kg diet	↓ TC, steatosis ↑ Faecal cholesterol	Trautwein et al., 2002

Clinical

Base Disease	**Supplementation**	**Effect**	**Reference**
Hyperlipidemia	300 mg oryzanol	↓TC, LDL-C, ApoB, TG, LPO ↑ HDL-C, ApoA II	Ishihara et al., 1984 Sasaki et al. 1990
Healthy	25 g triterpene alcohols and sterols	↓ Postprandial hyperglycaemia	Fukuoka et al., 2014
Healthy	2.1 g sterols	↓ TC, LDL-C	Vissers et al., 2000

Abbreviations: ACAT: acetyl-Coenzyme A acetyltransferase; **ACC**: acetyl-CoA carboxylase; **ACE**: angiotensin-converting enzyme; **ALP**: alkaline phosphatase; **ALT**: alanine aminotransferase; **ApoA**: apolipoprotein A; **ApoB**: apolipoprotein B; **AST**: aspartate aminotransferase; **BW**: body weight; **Cat**: catalase; **CK:** creatine kinase; **CPT1A**: carnitine palmitoyltransferase 1A; **CRP**: C-reactive protein; **eNOS**: endothelial nitric oxide synthase; **FAS**: fatty acid synthase; **G6Pase**: glucose 6-phosphatase; **G6PDH**: glucose 6-phosphatase; **GIP**: gastric inhibitory polypeptide; **GK**: glucokinase; **GPx**: glutathione peroxidase; **GR**: glutathione reductase; **GSH**: glutathione; **GST**: glutathione S-transferase; **HDL-C**: high-density lipoprotein cholesterol; **HMG-CoA**: 3-hydroxy-3-methylglutaryl-coenzyme A; **HO-1**: Heme oxygenase 1; **HOMA-IR**: homeostatic model assessment of insulin resistance; **HT**: hypertension; **IDL-C**: intermediate-density lipoprotein; **IL-1β**: interleukin 1 beta; **IL-6**: interleukin 6; **IR**: insulin resistance; **LDH**: lactate dehydrogenase; **LDL-C**: low-density lipoprotein cholesterol; **LPO**: lipid hydroperoxide; **NEFA**: non-esterified fatty acid; **NF-κβ**: nuclear factor kappa beta; **NO**: nitric oxide; **$NO_{(x)}$**: nitric oxide metabolites; **Nox-1**: NADPH oxidase 1; **$O_2^{\cdot-}$**: superoxide anion; **oxLDL**: oxidized low-density lipoprotein; **$p47^{phox}$**: neutrophil cytosol factor 1; **PEPCK**: phosphoenolpyruvate carboxykinase; **PON1**: paraoxonase 1; **PPAR**: peroxisome proliferator-activated receptor; **SOD**: superoxide dismutase; **SREBP**: sterol regulatory element-binding protein; **TBARS**: thiobarbituric acid reactive substances; **TC**: total cholesterol; **TG**:

triacylglycerol; **TGF-β**: transforming growth factor-β; **TNF-α**: tumour necrosis factor alpha; **VLDL-C**: very low-density lipoprotein cholesterol; **WAT**: white adipose tissue.

It has been shown that blood pressure was decreased in both, SHRSP (stroke-prone spontaneously hypertensive) rats and SHR (spontaneously hypertensive rats) through an ACE-related mechanism after short- and long-term treatments with ferulic acid (Ardiansyah et al., 2006; Ardiansyah et al., 2007; Ardiansyah et al., 2008; Ohara et al., 2009). In addition, Wang B et al. (2004) showed that sodium salt of ferulic acid inhibits platelet aggregation and prevents thrombus formation. Moreover, ferulic acid exhibited direct vasorelaxation activity and enhancement of the ACh-dependent relaxation in *ex vivo* rat aorta (Suzuki et al., 2007; Chen et al., 2009; Fukuda et al., 2015) as well as *in vivo* induction of eNOS (Senaphan et al., 2015) and vascular remodelling prevention (Senaphan et al., 2015; Wang et al., 2015).

Triterpene alcohols and sterols, which are found in rice bran alone or as a part of γ-oryzanol, stand out by their lipid-lowering activity. Studies in mice reported lower fatty acid synthesis in the liver and reduction of body weight through GIP-dependent and GIP-independent mechanisms (Fukuoka et al., 2014; Okahara et al., 2016), while in hamster stimulation of faecal cholesterol excretion through blockage of intestinal cholesterol absorption have been found (Trautwein et al., 2002). Moreover, triterpene alcohols and sterols from rice bran have been proved to ameliorate postprandial hyperglycaemia (Fukuoka et al., 2014) and sterol to reduce total and LDL cholesterol (Vissers et al., 2000) in humans.

5.2 Tocotrienols

Tocotrienols are members of the vitamin E family, which comprises four tocopherols and four tocotrienols. Structurally, they are composed of a chromanol ring and an aliphatic side chain. Tocotrienols occur naturally in low concentrations in certain grains and vegetables such as rye, wheat germ and barley. However, palm, soy bean and rice bran are exceptionally rich in tocotrienols (Adhikari et al., 2006). Table 6 summarizes the animal and clinical studies of tocotrienols-derived cardiometabolic protective effects. Traditionally, tocotrienols have been related to antioxidant activities through direct scavenging of reactive oxygen species and by upregulation of antioxidant enzymes (Siddiqui et al., 2010; Kanaya et al., 2004). Nevertheless, other activities which contribute to the protection of the vascular function include the inhibition of HMG-CoA reductase (Minhajuddin et al., 2005; Iqbal et al., 2003; Qureshi et al., 2000; Qureshi et al., 2001c), FAS (Qureshi et al., 2000), CPT1 and CYP7A1 enzymes (Burdeos et al., 2012) leading to lower blood cholesterol and lipid peroxidation (Table 6). The mechanisms proposed for the lipid lowering activity involve ubiquitination and degradation of HMG-CoA reductase and blockage of the processing of

sterol regulatory element-binding proteins (SREBPs) (Song & DeBose-Boyd, 2006). Rice bran tocotrienols also improved blood glucose and insulin release in animal studies (Siddiqui et al., 2010; Siddiqui et al., 2013; Qureshi et al., 2001c). Moreover, direct effects on eNOS activity were observed in SHR rats leading to reduce blood pressure (Newaz & Nawal, 1999; Newaz et al., 2003).

Tocotrienols differ structurally from tocopherols by the presence of three double bounds in the side chain, which confers higher antioxidant and anti-inflammatory activities, more efficient absorption and the possibility to be converted to tocopherols *in vivo*. It is noteworthy to mention that α-tocopherol not only did not exerted the same lipid lowering and antioxidant activities as tocotrienols, but also blocked the activity of the latter in terms of serum cholesterol and triacylglycerol lowering and mRNA expression of CPT1A and CYP7A1 in Fischer 344 rats fed a western diet (Shibata et al., 2016) with reproducible lipid lowering effects in humans (Table 6). The differential effects between tocopherols and tocotrienols could be due to the unsaturated side chain of tocotrienols which allows more efficient distribution in the cell membranes and penetration into tissues with saturated fatty layers such as the brain and liver (Suzuki et al., 1993).

Clinical studies focused the attention on hypercholesterolemia and diabetes mellitus type II. In the study performed by Baliarsingh S *et al.* (2005) the glucose metabolism was not improved because the medicated patients were glycaemically stable with glucose and HbA1c levels close to normal values. However, in this and other clinical studies rice bran tocotrienols improved lipid parameters, showing its interest in the prevention and treatment of hyperlipidaemia (Qureshi et al., 1997; O'Byrne et al., 2000; Qureshi et al., 2001d; Qureshi et al., 2002; Baliarsingh et al., 2005; Ajuluchukwu et al., 2007).

6. Conclusion

Rice bran and rice bran-derived products have been consumed for a long time owing to its functional properties, especially in Asia and North America, being regarded as value-added healthy products. The studies have demonstrated an inverse relationship between the intake of rice bran and products derived from rice bran and the rate of occurrence of risk factors for cardiovascular diseases. The health benefits of rice bran are though to arise mainly from bioactivities of γ-oryzanol, ferulic acid and tocotrienols. Rice bran and its main components have a variety of functional activities, including antioxidant, lipid lowering, anti-inflammatory, and anti-diabetic, among others. Ferulic acid and tocotrienols are known by its antioxidant activities while γ-oryzanol stands out by its lipid-lowering activities due to its sterol moiety.

Rice bran and rice bran oil are subjects of rancid reactions. Therefore, there is a need of stabilization of the product to allow its consumption. A number of methods have been developed to achieve lipase inactivation and often an increase in phytochemical concentration, despite biological activity being affected by temperature processes. That is the case of rice bran oil, where extraction processes increases the amount of phytochemicals in relation to raw rice bran but lowers its potential activity when heat is used in its production. Despite the technological limitations, most of the clinical studies summarised here were based on rice bran oil, which consistently proved protection on several cardiovascular disorders.

The range of doses used for the isolated molecules in relation to the average concentration present in rice bran or rice bran oil are comparable, both in animal and clinical studies. Also, there was a general good translational relationship between animal and clinical studies, despite some preliminary animal studies with rice bran where the dose was too high to be considered in a balanced human diet. However, most of the human studies have focus on the hypolipidemic and anti-diabetic effects, without inquiring in the mechanisms of action. In view of the limited number of clinical studies with rice bran extracts and the isolated active molecules, especially ferulic acid and γ-oryzanol, more studies are required to understand the individual contribution, potential synergistic effects and the influence of the rice bran matrix.

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8. Conflict of interest

None

9. References

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CAPÍTULO II

No he fracasado. He encontrado 10.000 maneras que no funcionan

(Thomas Alva Edison, 1847-1931)

EL EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ REDUCE EL DESARROLLO DE LA PLACA ATEROSCLERÓTICA Y DE LA ESTEATOSIS EN RATONES APOE-/- ALIMENTADOS CON UNA DIETA ALTA EN GRASA

Perez-Ternero C, Claro C, Parrado J, Alvarez de Sotomayor M, Herrera MD

En revisión en *Nutrition*

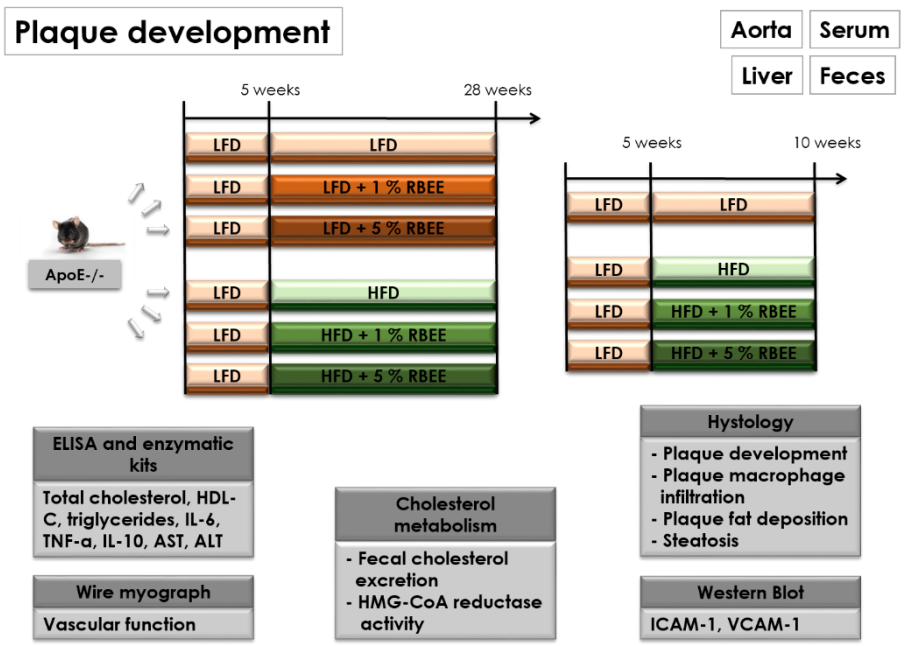
El salvado de arroz es un subproducto de la molienda del arroz rico en moléculas bioactivas como el ácido ferúlico, el γ-oryzanol, los fitosteroles, los tocotrienoles y los tocoferoles. Estas moléculas confieren al salvado de arroz propiedades hipolipemiantes, antioxidantes, antiinflamatorias y antidiabéticas, entre otras. Sin embargo, a pesar de su potencial para la prevención de enfermedades cardiovasculares, el salvado de arroz se destina fundamentalmente a la alimentación del ganado ya que tiene gran tendencia al enranciamiento y una naturaleza oleosa que no lo hace interesante para la industria alimentaria. Dado que el extracto enzimático de salvado de arroz (EESA) ha demostrado previamente sus propiedades hipolipemiantes, antioxidantes y antiinflamatorias, el objetivo de este trabajo fue el estudio del mecanismo hipolipemiante y de los efectos de la suplementación de la dieta con EESA en el desarrollo de la placa aterosclerótica, de la disfunción vascular y de hígado graso en ratones deficientes en apolipoproteína E (ApoE-/-). Asímismo, se planteó como objetivo el estudio de los efectos de la composición grasa y alto contenido en colesterol de la dieta en la actividad derivada de la suplementación con EESA.

Ratones ApoE-/- de 5 semanas de edad fueron alimentados durante 23 semanas con una dieta baja en grasa (LFD) o alta en grasa y colesterol (HFD), suplementada o no al 1 o 5% con EESA. La semana previa al sacrificio, se recolectaron las heces emitidas durante un periodo de 24 h y al finalizar el tratamiento, los ratones se sacrificaron y se extrajeron aorta, hígado y suero.

La suplementación de la dieta HFD con EESA a las dosis ensayadas, aumentó los valores séricos de colesterol HDL y previno el incremento de colesterol total y de alanina aminotransferasa inducido por el mayor contenido en grasa y colesterol del pienso. En estos animales, el suplemento de EESA redujo la actividad de HMG-CoA-Reductasa (1 y 5% EESA) y aumentó la excreción fecal de colesterol (5% EESA). Independientemente del contenido graso de la dieta, la suplementación al 5% EESA redujo el desarrollo de la placa aterosclerótica, mientras que sólo en los ratones alimentados con la dieta HFD se redujo la

deposición de lípidos, la infiltración de macrófagos en la válvula aortica y la expresión de las moléculas de adhesión ICAM-1 y VCAM-1. Finalmente, la suplementación con EESA redujo la esteatosis en los ratones con dieta LFD (1% EESA) y HFD (5% EESA).

Con todo esto, podeos concluir que el consumo regular de EESA en una dieta alta en grasa y colesterol reduce el desarrollo de la placa aterosclerótica y de hígado graso por medio de efectos antiinflamatorios e hipolipemiantes derivados de la disminución de la actividad de la enzima HMG-CoA-Reductasa y del incremento de la excreción fecal de lípidos.



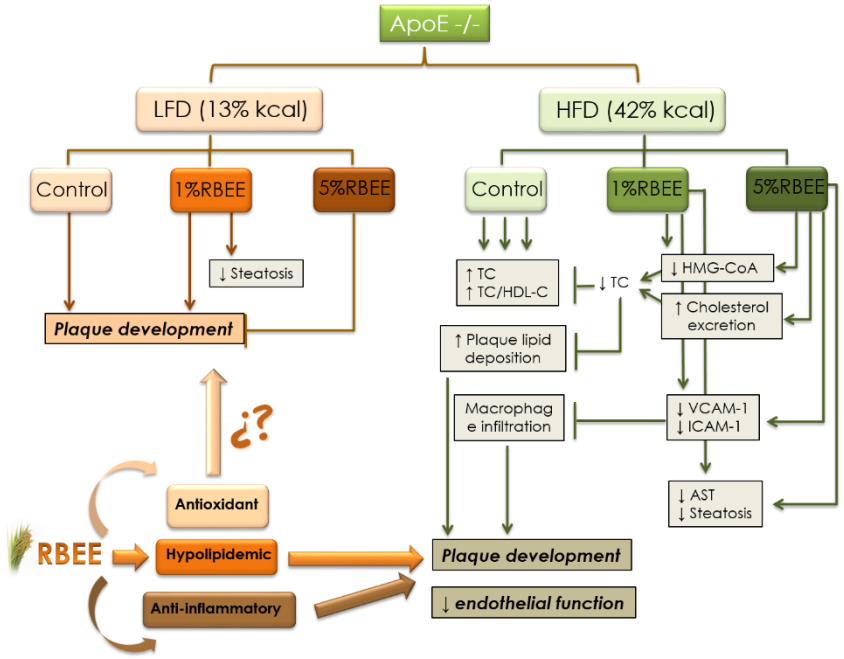


Figura 16: Diseño experimental y resumen de resultados.

RICE BRAN ENZYMATIC EXTRACT REDUCES ATHEROSCLEROTIC PLAQUE DEVELOPMENT AND STEATOSIS IN HIGH FAT FED APOE-/- MICE

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Abstract

Objective: Rice bran is a by-product of rice milling rich in bioactive molecules such as γ-oryzanol, phytosterols and tocotrienols. The rice bran enzymatic extract (RBEE) previously showed vessel remodelling prevention and lipid lowering, antioxidant, anti-inflammatory and antiapoptotic activities. Here, we aimed to identify RBEE hypolipidemic mechanism and to study the effects of RBEE on the progression of atherosclerosis disease and linked vascular dysfunction and liver steatosis in ApoE-/- mice fed low (LFD) or high (HFD) fat diets.

Research Methods & Procedures: ApoE-/- mice were fed LFD (13% kcal) or HFD (42% kcal) supplemented or not with 1 or 5% RBEE (w/w) for 23 weeks. Then, serum, aorta, liver and faeces were collected and flash frozen for further analysis.

Results: RBEE supplementation of HFD improved serum values by augmenting HDL-C and preventing total cholesterol and AST increase. HMG-CoA reductase activity was attenuated (1% and 5% RBEE) and cholesterol excretion increased (5% RBEE). 5% RBEE diet supplementation reduced plaque development regardless of the diet. In HFD-fed mice, both doses of RBEE reduced lipid deposition and macrophage infiltration in the aortic sinus and downregulated ICAM-1 and VCAM-1 expression. None of these effects were observed in mice fed LFD. Liver steatosis was reduced by RBEE supplementation of LFD (1% RBEE) and HFD (1 and 5% RBEE) and nuclear PPAR-α expression upregulated in HDF 5% RBEE group.

Conclusion: Regular consumption of RBEE-supplemented HFD reduced plaque development and liver steatosis by decreasing inflammation and hyperlipidemia through a HMG-CoA reductase activity and lipid excretion related mechanism.

Keywords: atherosclerosis; rice bran enzymatic extract; HMG-CoA; macrophage infiltration; adhesion molecules; steatosis.

1. Introduction

Atherosclerosis is a state of chronic vascular inflammation which leads to the onset of cardiovascular diseases, the main cause of death worldwide. Of special importance in its pathogenesis are the elevation of low-density lipoproteins (LDL) and its oxidation to oxLDL which triggers foam cell formation leading to fatty streak lesion development [1] and endothelial dysfunction [2]. Those pro-inflammatory stimuli promote adhesion molecules expression and leukocyte rolling and firm adhesion to the arterial wall [3].

Diet patterns are pivotal issues within the controllable cardiovascular risk factors. Reduction of saturated fat and cholesterol intake are the initial therapeutic approaches for mild cholesterol rates and background tools in any case. In addition to a cardiovascular-healthy diet, popularity of functional foods has grown recently [4]. Among them, rice bran and rice bran oil have demonstrated hypocholesterolemic, antioxidant, antidiabetic and anti-inflammatory activities in humans and rodents [5]. Rice bran is a by-product of rice milling which comprises the outer layers of rice kernel, the aleurone and the embryo [6]. These layers are rich in unsaturated (oleic and linoleic acids) fatty acids and high value proteins. However, the hypolipidemic, antioxidant and anti-inflammatory potential activity of rice bran relies on the unsaponifiable fraction, consisting of tocols (tocopherols and tocotrienols), γ-oryzanol, triterpene alcohols and phytosterols [7]. In spite of its interesting composition, raw rice bran and rice bran oil use by the food industry are limited because of their water insolubility and rapid rancidity due to lipase activity.

The novel rice bran enzymatic extract (RBEE) used in this work avoids these weaknesses giving rise to a syrup completely water soluble and rancidity protected as a result of the endoprotease extraction process. This enzymatic method inactivates lipases responsible for lipid degradation and disperses cells and aggregates leading to liberation and enrichment in the bioactive components (i.e. γ-oryzanol and tocotrienols). The final product is water soluble due to peptide-interaction of the hydrophobic components [8]. RBEE has previously shown protective effects on small mesenteric arteries remodelling and endothelial function as well as hyperlipidemia prevention and oxidative stress, inflammation and apoptosis reduction in the aorta of apolipoprotein E-knockout (ApoE-/-) mice [9-11].

The aims of this study were to investigate mechanisms of the hypolipidemic action and the effects of RBEE dietary supplementation on atherosclerotic plaque development, vascular function and liver steatosis in apolipoprotein E-knockout mice (ApoE-/-). The effects of RBEE were tested on mice fed high and low fat diets in order to identify whether RBEE activity depends on dietary cholesterol and saturated fat consumption.

2. Material and methods

2.1 Animals and diets

From five weeks of age, male ApoE-/- mice on a C57BL/6J background (Charles River Laboratories, L'Abresle, France) were randomly separated into six groups of treatment (n = 15). Three groups were fed low fat diet (LFD, 2014, Teklad, Envigo, Madison, USA) containing 13% fat (% kcal), supplemented or not with either 1% RBEE (LFD 1%) or 5% RBEE (LFD 5%). The last three groups were fed HFD containing 0.15% (w/w) cholesterol and 42% (% kcal) fat (HFD, TD 88137, Teklad, Envigo, Madison, USA) supplemented or not with 1% RBEE (HFD 1%) or 5% RBEE (HFD 5%). Composition of diets, RBEE and raw rice bran are shown in Table 1. RBEE concentrations were those that had shown antioxidant and anti-inflammatory properties in previous studies [9-13]. At 28 weeks of age, mice were anesthetized with phenobarbital and sacrificed by cardiac puncture and exsanguination. All experimental procedures were approved by University of Seville (Spain) Committee for ethical experimentation.

2.2 Rice bran enzymatic extract

RBEE was prepared by enzymatic hydrolysis as previously described [8], through hydrolysis with a trypsin- and chymotrypsin-like endoprotease mixture (Bioproteasa LA450, Biocon Española, Spain) in a bioreactor pH (pH 8) and temperature (60 °C) controlled. Rice bran macro and micronutrient composition was characterized as previously described [8] and is provided in Table 1.

2.3 Serum lipids, cytokines and transaminases analysis

Serum was obtained by centrifugation (20 minutes, 4 000 g, RT). Serum total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST) (Spin React, CIMA Diagnostics, Girona, Spain) and triglycerides (TG, WAKO Diagnostics, Richmond, USA) were quantified using colorimetric kits based on enzymatic reactions. Serum cytokines (TNF-α, IL-6 and IL-10) were determined by ELISA (R&D Systems, Inc., Minneapolis, USA), according to the manufacturer's instructions.

2.4 HMG-CoA reductase activity in the liver

HMG-CoA reductase activity was measured in liver homogenates according to the method described by Venugopala and Ramakrishnan [14]. The ratio of HMG-CoA to mevalonate was taken as an index of HMG-CoA reductase activity, with a low ratio

indicative of high enzyme activity and vice versa. The absorbance due to the formation of hydroxamic acid complexes with ferric salts was read in a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, USA).

Table 1: Composition of the experimental diets, RBEE and RB

Nutrient	LFD	LFD 1%	LFD 5%	HFD	HFD 1%	HFD 5%	RBEE	RB
Energy (kcal/g)	2.90	2.92	3.00	4.50	4.50	4.52	5.49	4.75
Protein (% kcal)	20.0	20.1	20.6	15.2	15.4	16.0	24.7	14.3
Carbohydrate (% kcal)	67.0	66.5	64.2	42.7	42.4	41.2	21.1	43,0
Fat (% kcal)	13.0	13.4	15.2	42.0	42.1	42.7	54.2	42.6
Saturated	15.0	15.1	15.3	61.8	61.4	59.7	20.4	18.3
Monounsaturated	17.5	17.7	18.7	27.3	27.5	28.1	42.4	43.1
Polyunsaturated	52.5	52.3	51.7	4.70	5.01	6.26	35.8	37.2
Unknown	15.0	14.9	14.3	6.20	6.09	5.94	1.40	1.34
Cholesterol (g/100 g)	-	-	-	0.15	0.15	0.14	-	-
Phytosterols (mg/kg)	-	35.53	177.7	-	35.53	177.7	3553	4140
γ-oryzanol (mg/kg)	-	89.5	447.5	-	89.5	447.5	8950	7100
Tocotrienols (mg/kg)	-	1.7	8.5	-	1.7	8.5	170	137
Tocopherols (mg/kg)	108	108	107.3	219	217.7	212.7	93.4	125

2.5 Faecal lipid excretion

Faecal lipids were extracted using the method described by Folch et al. [15]. TC was extracted from 100 mg of faeces with a chloroform-methanol (2:1) solution and measured using a commercial kit (Spin React, CIMA Diagnostics, Girona, Spain).

2.6 Vascular reactivity

To test endothelium dependent relaxation, proximal descending aorta rings were mounted in a wire myograph (Danish MyoTechnology, Aarhus, Denmark) as previously described [16]. Vessels contracted to the 80% of the maximum capacity with the thromboxane receptor agonist 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α

(U46619) were exposed to cumulative concentrations of acetylcholine (ACh, 0.001–10 μmol/L).

2.7 Histological analysis of atherosclerotic lesions in the aortic sinus and liver steatosis

Atherosclerotic lesions in the aortic sinus were quantified in 10 μm transverse sections of 4% paraformaldehyde-fixed hearts. Averages of the sum of total positive atherosclerotic plaque areas were calculated from 3 serial Oil-Red-O stained sections in the middle of the aortic valve cusps. Staining for lipid depositions was performed with Oil-Red-O and macrophage infiltration was visualized by immunohistochemistry staining for Mac-3 (eBioscience, San Diego, USA) and expressed as the ratio of positive area to total cross-sectional vessel wall area. For liver steatosis quantification, 10 μm sections of frozen livers were stained with Oil-Red-O, as previously described [17]. All images were recorded with an Olympus BX61 microscope and positive areas quantified using ImageJ v1.45 software (NIH, USA).

2.8 Western Blotting

Thoracic and abdominal aorta were excised and homogenized in lysis buffer [13, 16]. Nuclear extraction from liver tissue was performed with a commercial kit (Active Motif, Rixensart, Belgium), as recommended by the manufacturer. Equal amounts of protein were resolved on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane and incubated with the following primary antibodies: anti-VCAM-1, anti-ICAM-1 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany) or anti-PPAR-α (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany). Antibody binding was visualized using an enhanced chemioluminescence detection system (Fujifilm LAS-3000 Imager; Stamford, USA) and densitometric analysis was performed with ImageJ v1.45 software (NIH, USA) using β-actin as internal control to verify equal protein loading.

2.9 Statistical analysis

Data were expressed as mean±SEM and n reflects the number of assays conducted. Two-way ANOVA analysis was performed, followed by Newman-Keuls test. All statistical computations in this paper have been performed using programs written in the R language [18]. Results with $p<0.05$ were considered statistically significant.

3. Results

3.1. Effect of RBEE on serum lipid profile, systemic cytokines and transaminases

Serum lipid and cytokine concentrations are shown in Table 2. HFD increased fasting serum concentrations of TC ($p<0.001$) and TC/HDL-C ratio ($p<0.05$) and reduced HDL-C ($p<0.001$) compared to LFD.

1 and 5% RBEE supplementation of HFD prevented the increase in TC and augmented HDL-C ($p<0.01$). Consequently TC/HDL was restored towards values found in LFD-fed mice. None of these values were modified by the introduction of RBEE in LFD.

In the absence of RBEE, HFD resulted in higher TG serum concentration than non-supplemented LFD ($p<0.01$). Despite the fact that TG concentration of 1 and 5% RBEE-supplemented HFD did not differ statistically from non-supplemented HFD, this endpoint neither differed from control LFD-fed mice.

Additionally, pro-inflammatory cytokines IL-6 and TNF-α, and the anti-inflammatory IL-10 were upregulated by HFD compared to LFD ($p<0.001$). RBEE supplementation of HFD tended to reduce TNF-α, without reaching statistical significance compared to non-supplemented HFD.

Liver enzymes ALT and AST were upregulated by HFD indicating liver inflammation ($p<0.01$ and $p<0.05$ for ALT and AST, respectively). Although ALT levels were not modified by diet supplementation with RBEE, AST release from the liver was reduced in HFD fed animals ($p<0.05$), in contrast to LFD.

Table 2: Serum lipid and cytokine profile.

	LFD	LFD 1%	LFD 5%	HFD	HFD 1%	HFD 5%
TC (mg/dL)	415.9 ± 20.1 ^{c}	387.1 ±31.7 ^{c}	405.0 ± 29.5 ^{c}	956.5 ± 63.6 ^{a}	751.7 ± 72.1 ^{b}	714.0 ± 59.6 ^{b}
HDL-C (mg/dL)	43.2 ± 5.6 ^{b}	41.0 ± 3.6 ^{b}	44.3 ± 4.6 ^{b}	34.6 ± 6.1 ^{b}	64.7 ± 9.0 ^{a}	68.6 ± 7.6 ^{a}
TC/HDL-C	10.6 ± 1.5 ^{b}	9.3 ± 1.0 ^{b}	10.1 ± 1.2 ^{b}	20.8 ± 2.4 ^{a}	13.4 ± 1.6 ^{b}	12.9 ± 1.5 ^{b}
TG (mg/dL)	78.9 ±6.7 ^{b}	82.2 ± 14.0 ^{b}	112.9 ± 8.9 ^{ab}	155.0 ±19.6 ^{a}	130.4 ± 23.7 ^{ab}	101.4 ± 17.0 ^{ab}
IL-6 (pg/mL)	42.9 ± 1.9 ^{b}	40.5 ± 2.2 ^{b}	48.9 ± 3.2 ^{b}	59.2 ± 5.9 ^{a}	61.6 ± 6.2 ^{a}	54.9 ± 3.2 ^{a}
TNF-α (pg/mL)	30.4 ± 1.4 ^{c}	33.6 ± 2.0 ^{bc}	34.0 ± 2.3 ^{bc}	42.9 ± 1.6 ^{a}	38.4 ± 1.4 ^{ab}	37.8 ± 1.6 ^{ab}
IL-10 (pg/mL)	35.3 ± 1.4 ^{b}	39.2 ± 2.4 ^{b}	36.5 ± 2.1 ^{b}	43.3 ± 2.6 ^{a}	42.7 ± 2.5 ^{a}	49.0 ± 2.9 ^{a}
ALT (U/L)	77.0 ± 29.5 ^{b}	35.0 ± 31.6 ^{b}	37.9 ± 36.3 ^{b}	157.5 ± 25.1 ^{a}	94.5 ± 27.5 ^{ab}	164.1 ±29.8 ^{ab}
AST (U/L)	44.5 ± 7.7 ^{b}	45.5 ± 3.5 ^{b}	29.9 ± 15.2 ^{bc}	79.6 ± 10.7 ^{a}	10.0 ± 9.6 ^{c}	13.56 ± 6.8 ^{c}

3.2. RBEE regulated HMG-CoA reductase activity and lipid faecal excretion

HMG-CoA reductase activity was reduced in mice fed HFD supplemented with either 1 or 5% RBEE (Fig.1 A; $p<0.05$). Moreover, increased faecal excretion of TC was enhanced by 5% RBEE supplementation of HFD (Fig.1 B; $p<0.05$). However, these two parameters were not modified by the introduction of RBEE in LFD.

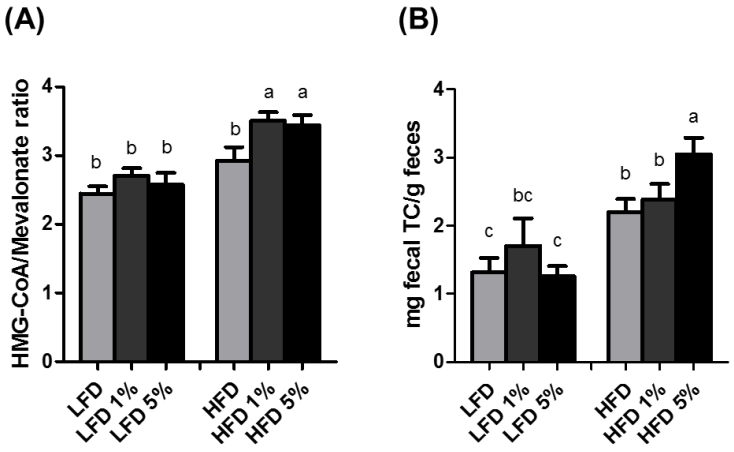

Figure 1: HMG-CoA reductase activity and faecal lipids excretion. (**A**) HMG-CoA/Mevalonate ratio was taken as an index of HMG-CoA reductase activity in the liver. (**B**) Total cholesterol (TC) eliminated in faeces. Values were mean±SEM (n=8). Columns with different letters differ significantly (two-way ANOVA followed by Newman-Keuls test).

3.3. RBEE did not improve endothelial dysfunction

HFD reduced ACh-induced relaxation (Fig.2; Emax (%), LFD: 72.76±2.3 and HFD: 56.2±2.0, $p<0.05$). RBEE did not prevent endothelial dysfunction (Emax (%), LFD 1%: 67.50±2.9, LFD 5%: 62.35±2.4, HFD 1%: 45.91±3.5 and HFD 5%: 48.13±5.3). Animals fed LFD responded to lower concentrations of ACh (pD2 (M), LFD: 8.02±0.53 and HFD: 6.65±0.17, $p<0.001$). The addition of RBEE to LFD did not modify this response (pD2 (M), LFD 1%: 7.98±0.26, LFD 5%: 7.91±0.48, HFD 1%: 6.79±0.28 and HFD 5%: 6.40±0.48).

To identify if RBEE could prevent vascular impairment in younger mice, vascular function of 10 weeks old mice fed HFD supplemented or not with 1 or 5% RBEE or LFD was assessed (Supplementary Fig.1). As in older mice, HFD diet induced endothelium impairment in 10-week old mice (Supplementary Fig.1; $p<0.001$). However, RBEE diet supplementation protected endothelium dependent relaxation ($p<0.001$ vs HFD).

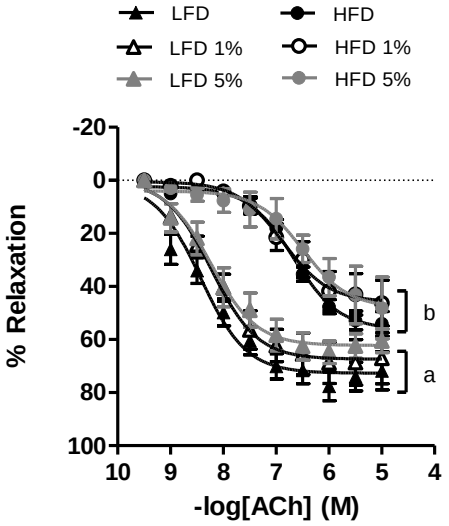

Figure 2: RBEE did not improve endothelial dysfunction. Concentration-response curves to ACh of aortic ring from ApoE-/- mice fed low (LFD) or high (HFD) fat diet supplemented or not with 1 or 5% RBEE. Values were mean±SEM (n=5-7). Groups with different letters differ significantly (two-way ANOVA followed by Newman-Keuls test).

3.4. Histological analysis of atherosclerotic lesions

Although all experimental groups showed atherosclerotic plaques in the aortic sinus regardless of the diet, HFD-fed animals developed more extensive atherosclerotic lesions than those fed LFD (Fig.3 A; $p<0.001$). Plaque formation was attenuated by 5% RBEE supplementation of both HFD and LFD (Fig.3 A).

Independently of the diet, all ApoE-/- groups showed macrophage infiltration (Fig.3 B and D) and lipid deposition in the aortic sinus (Fig.3 C and E). Aortic sinus of HFD-fed mice exhibited enhanced lipid deposition ($p<0.001$).

RBEE supplementation of HFD attenuated macrophage infiltration by 27.1% and 47.8% for HFD 1% and HFD 5%, respectively (Fig.3 B and D). In addition, RBEE added to HFD sharply attenuated lipid accumulation by 35.4% and 59.17% for HFD 1% and HFD 5%, respectively (Fig.3 C and E). Conversely, supplementation of LFD with RBEE had no effect in macrophage infiltration or lipid deposition.

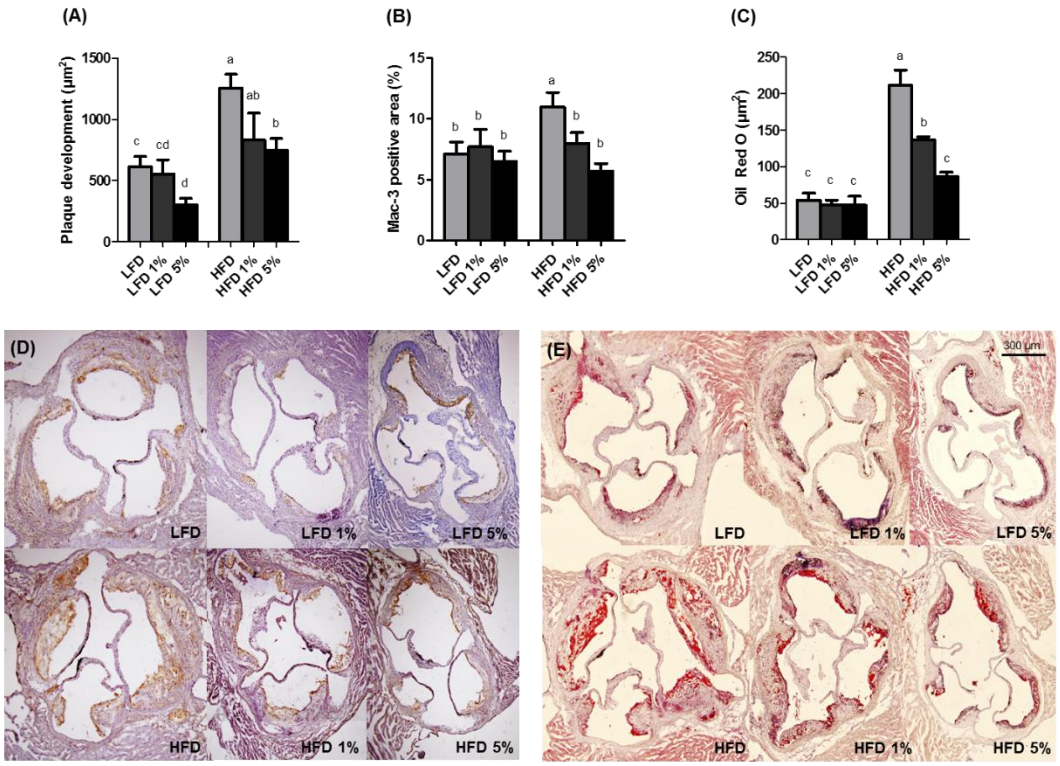

Figure 3: RBEE attenuated plaque development, macrophage infiltration and lipid deposition in the aortic sinus. (A) Quantification of plaque development of aortic sinus from ApoE-/- mice fed low (LFD) or high (HFD) fat diets supplemented or not with 1 or 5% RBEE. (B) Quantification and (D) representative images of immunostaining for Mac-3. (C) Quantification and (E) representative images of Oil-Red-O-staining. Values were mean±SEM (n=8). Columns with different letters differ significantly (two-way ANOVA followed by Newman-Keuls test). Magnification 4X, scale bar=300 µm.

3.5. VCAM-1 and ICAM-1 expression was attenuated by RBEE while PPAR-α was increased

As shown in Fig.4, VCAM-1 and ICAM-1 expressions in whole aortic tissue increased with HFD by 157.7±17.8% and 156.0±19.3% for VCAM-1 and ICAM-1 ($p<0.001$), respectively. Expressions of both adhesion molecules decreased with RBEE supplements ($p<0.05$), reaching expression levels of mice fed LFD. In the liver, 5% RBEE supplementation increased nuclear expression of PPAR-α by 174.6±24.6%. The expression of these molecules in mice fed RBEE-supplemented LFD remained unchanged.

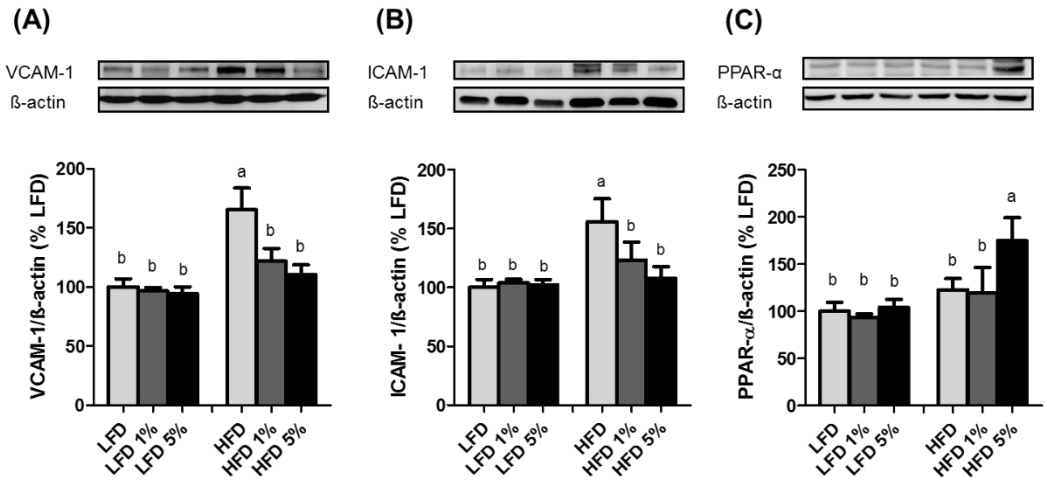

Figure 4: RBEE downregulated ICAM-1 and VCAM-1 expression. Protein expression of (A) VCAM-1 and (B) ICAM-1 in the aorta and (C) PPAR-α in the liver of ApoE-/- mice fed low (LFD) or high (HFD) fat diet supplemented or not with 1 or 5% RBEE. Values were mean±SEM (n=10). Columns with different letters differ significantly (two-way ANOVA followed by Newman-Keuls test).

3.6. RBEE reduced liver steatosis

Fig. 5 shows representative pictures and quantification of the percentage of Oil-Red-O positive area in liver. ApoE-/- mice showed the typical liver steatosis, which was reduced by RBEE supplementation of LFD (1% dose, $p<0.05$) and HFD (1 and 5% doses, $p<0.001$). 1% RBEE dose was more effective in the reduction of liver steatosis.

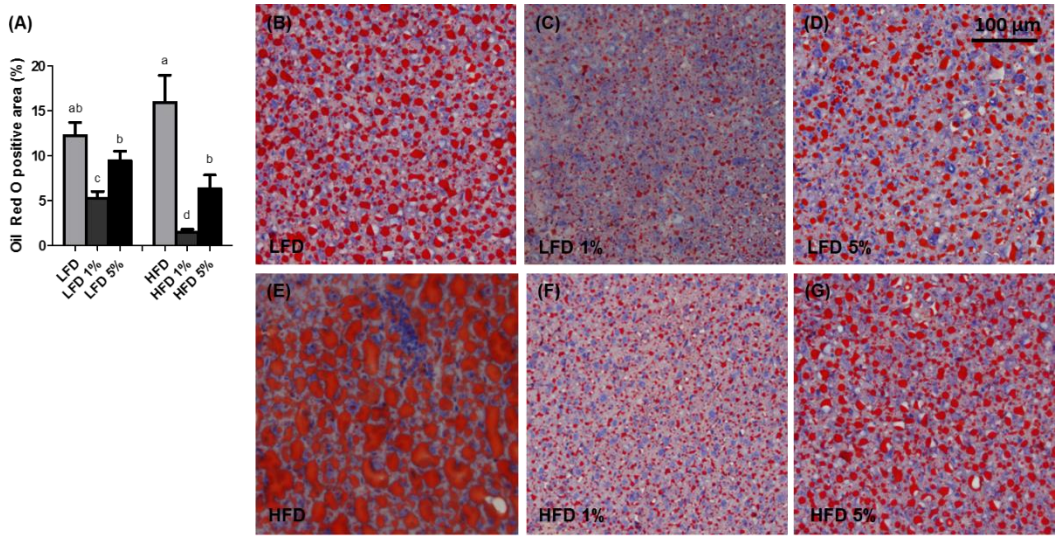

Figure 5: RBEE reduced liver steatosis. (**A**) Quantification of Oil-Red-O positive areas (%) of 10 µM liver section from ApoE-/- mice fed low (LFD, **B-D**) or high (HFD; **E-G**) diets. Values were mean±SEM (n=6). Columns with different letters differ significantly (two-way ANOVA followed by Newman-Keuls test). Magnification 10X, scale bar=100 µm.

4. Discussion

In our previous works, RBEE had shown a broad spectrum of vascular activities including vessel remodelling prevention, anti-inflammatory activities in vitro and in vivo and reduction of hyperlipidemia, oxidative stress and apoptosis. Therefore, the aims of this work were first, to examine whether RBEE diet supplementation could result in the prevention of atherosclerosis development, vascular dysfunction and liver steatosis in ApoE-/- mice and second, to investigate the hypolipidemic mechanism. The study of the effects of RBEE on mice fed LFD and HFD aimed to identify different outcomes depending on the diet composition. As expected, RBEE supplementation of HFD attenuated plaque development, lipid deposition, macrophage infiltration and liver steatosis. RBEE also prevented protein expression of the adhesion molecules ICAM-1 and VCAM-1 in the aorta and upregulated the expression of PPAR-α in the liver. Nevertheless, the systemic pro-inflammatory markers were not significantly reduced. In addition, supplementation with the extract enhanced faecal cholesterol excretion and reduced HMG-CoA reductase activity. Both mechanisms and may contribute to explain serum cholesterol reduction.

As in our previous studies, serum lipid pattern was improved by RBEE supplementation in HFD-fed mice. However, LFD groups remained unchanged, suggesting for the first time that diet composition could be critical for RBEE effectiveness in lowering cholesterol. Therefore, the lower effects observed in animals fed LFD could be due to cholesterol absence in this diet, highlighting the importance of RBEE phytosterols and triterpenic alcohols participation in cholesterol metabolism. Phytosterols are cholesterol uptake disruptors in the gut. Their properties have been attributed to a physicochemical interference with micellar solubilization of cholesterol in the gut lumen [19], although a more complex mechanism involving liver X receptor activation and expression of the basolateral sterol exporter ABC-A1 has been proposed as well [20]. The amount of phytosterols in the diets negatively correlates with cholesterol absorption in a dose-dependent manner [21]. Phytosterols are present in the RBEE in its free form and as a part of the γ-oryzanol molecule. Several groups failed to find γ-oryzanol in serum after consumption due to low permeation of sterol ferulates across the gut [22]. However, γ-oryzanol has been proposed to be hydrolysed, liberating ferulic acid and triterpenic alcohols as free molecules [22], releasing the complementary hypolipidemic activities in the gut (phytosterols) and systemic antioxidant and anti-inflammatory activities after absorption (ferulic acid) [23]. Higher concentration of these molecules in the 5% supplemented diets could partially explain the more robust effects observed with this dose.

Moreover, apart from increasing cholesterol excretion, rice bran bioactive components (i.e. phytosterols, ferulic acid, tocotrienols and γ-oryzanol) have been reported to reduce cholesterol synthesis through HMG-CoA reductase inhibition [24]. We found that RBEE was able to reduce HMG-CoA reductase activity in mice fed HFD. Similarly, a previous research described that another rice bran enzymatic extract was able to decrease plasma cholesterol in Wistar rats fed high-fat, high-cholesterol diet by means of decreasing both intestinal cholesterol absorption and HMG-CoA reductase activity [25]. Conversely, we did not find the decrease in HMG-CoA hepatic activity due to HFD described by Wang et al [25]. This difference could be explained not only by intrinsic species-related characteristics but also as a result of ApoE deletion on cholesterol metabolism since ApoE-/- mice fed LFD exhibit half HMG-CoA reductase activity but higher plasma cholesterol than their wild type counterparts [26].

Of special interest in the present work is the increase of HDL-C in HFD treated groups, which is known to be related to reverse cholesterol transport and lower atherogenesis risk [27], and the upregulation of PPAR-α in the liver of mice fed HFD 5% RBEE, which is related to anti-inflammatory activities and to fatty acid oxidation in response to energy demand [28]. A recent study reported increased PPAR-γ expression in adipocytes treated with γ-oryzanol [29], while a number of phytochemicals, such as polyphenols, terpenoids and phytosterols, have been related to upregulation of both PPAR-α and PPAR-γ, showing beneficial effects on diabetes, obesity and dyslipidaemia [30, 31].

We previously confirmed that lower concentrations of serum cholesterol induced by RBEE consumption rebound to a reduction of oxLDL [9], whose accumulation in the arterial wall is known to induce monocyte and leukocyte infiltration. Although RBEE only showed a trend of improvement on circulating cytokines, diet supplementation strongly reduced local vascular inflammation in HFD-fed animal as revealed by reduced expression of ICAM-1 and VCAM-1 and lower macrophage infiltration. Moreover, in our previous studies RBEE diet supplementation reduced NF-κβ activation and the expression of TNF-α, iNOS and COX-2 in the aorta of ApoE-/- mice [9]. Adhesion molecules play a critical role in monocyte adhesion and infiltration into the arterial wall, where monocyte differentiate into macrophage and become foam cells, contributing to the progression of the atherosclerotic plaque. Monocyte and macrophage phenotype greatly affects the progression of the atherosclerotic plaque. As we explained elsewhere, RBEE induced anti-inflammatory monocyte phenotype and a shift towards M2 macrophages, reducing the release of TNF-α and IL-6 pro-inflammatory cytokines [9]. The RBEE phenolic molecule ferulic acid was identified as the main responsible for these actions. Supporting our results, others previously reported anti-inflammatory effects

of rice bran bioactive components and by-products in vitro and in different rodent models [7, 32, 33].

High plasma lipoprotein concentration compromises endothelial function [34], being endothelial dysfunction an early predictor of atherosclerosis. Moreover, atherosclerotic plaques impair local eNOS activity and prooxidant environment leads to NO consumption by oxygen reactive species, reducing NO-dependent vasodilatation. Despite the reduction of plaque development of all HFD-treated groups, endothelial dysfunction was not restored. We also conducted experiments in mice aged 10 weeks (Supplementary Fig.1). In contrast to 28-week old mice, endothelial function was protected by RBEE in younger mice. We and others previously found endothelial function improvement by rice bran extracts in ApoE-/- mice as well as in obese and hypertensive rats as a result of reduced oxidative stress, eNOS upregulation and anti-inflammatory effects [13, 35]. Interestingly, the ACh-induced relaxation was restored in mesenteric arteries of 30-week old ApoE-/- mice fed HFD supplemented with RBEE. The protective effect was related to lower eNOS phosphorylation in the inhibitory site Thr495, which augmented the availability of NO [10]. Therefore, along with the antioxidant and anti-inflammatory effects, eNOS upregulation may be the mechanism involved in the protective effect. Atherosclerotic plaque progression and consequent increase of oxidative stress in older mice may account for the loss of endothelial protection, in comparison with mesenteric arteries, which do not develop atherosclerotic plaques and younger mice, whose plaque is smaller.

As a side-effect of hypercholesterolemia, ApoE-/- mice develop non-alcoholic fatty liver, which contributes to atherosclerosis development through release of atherogenic factors such as very low density lipoproteins (VLDL), proinflammatory cytokines and plasminogen activator inhibitor-1 [36]. The release of AST and ALT liver enzymes indicates inflammation and liver damage. Here, RBEE diet supplementation only reduced the release of AST, which in contrast to ALT is not liver specific and also reflects inflammation of other tissues. RBEE diet supplementation reduced liver steatosis, which could be related to one of its components, ferulic acid and its metabolite caffeic acid, which have been reported to regulate triglyceride liver synthesis and the expression of genes related to triglyceride and cholesterol metabolism (i.e. FAS, DGAT-2, ATGL, PPAR-α, SREBP-2, LXR-α, LDL-R) [37]. Also, long-term consumption phytosterols, another component of RBEE, was reported to reduce steatosis and hyperlipidaemia due to higher faecal cholesterol excretion, and to restore the expression of PPAR-α, CPT-1α and PCK-1 in the liver [31]. In our study, 1% RBEE prevention of liver steatosis was more effective than 5% RBEE, in contrast to the effects upon serum cholesterol. This effect could be explained by the higher supply of tocotrienols and tocopherols in 5% RBEE dose. Tocotrienols can be converted to tocopherols in vivo, which

not only do not show the same antioxidant and lipid lowering properties of tocotrienols, but also impede their performance when supplied in high amounts [38].

Although most of the effects of RBEE were only found in HFD, 5% RBEE supplementation of LFD-fed mice reduced atherosclerotic plaque size in the aortic sinus. This result cannot be explained by reduction of lipid deposition or lower macrophage infiltration, which remained unchanged. RBEE has previously shown powerful antioxidant activity capable to reduce superoxide anion production and to down-regulate NADPH oxidase expression of HFD-fed ApoE-/- mice and of an animal model of metabolic syndrome fed LFD [9, 13], but more research is needed to confirm if its antioxidant activity contributes to the effect of RBEE in atherosclerosis development independently of the cholesterol reduction.

In conclusion, chronic consumption of RBEE in high fat diets can help to ameliorate serum lipid profile, reducing atherosclerotic plaque development and liver steatosis. Disruption of intestinal cholesterol absorption and inhibition of HMG-CoA reductase activity were identified as the main mechanisms of RBEE in HFD-fed animals. Thus, joining the technological advantages with the in vivo activities, RBEE is presented as an interesting ingredient for nutraceutical products. However, further studies should be conducted to elucidate the mechanisms underlying RBEE activities, giving light to the bioavailability and metabolism of its bioactive components to warranty safe human consumption in terms of toxicity.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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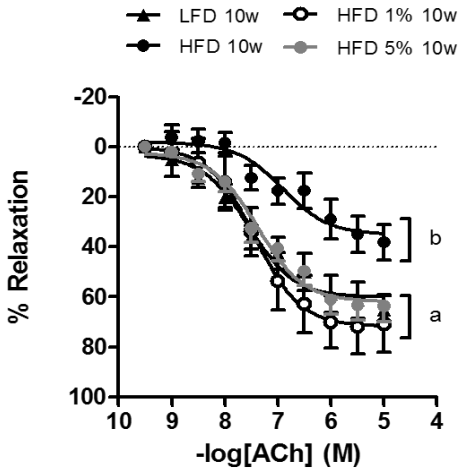

Supplementary Figure 1: RBEE restored endothelial dilatation in young animals. Concentration-response curves to ACh of aortic ring from ApoE-/- mice fed low (LFD) or high (HFD) fat diet supplemented or not with 1 or 5% RBEE. Values were mean±SEM (n=5-7). Groups with different letters differ significantly (two-way ANOVA followed by Newman-Keuls test).

CAPÍTULO III

El mayor error que una persona puede cometer es tener miedo de cometer un error

(Elbert Hubbard, 1856-1915)

EL EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ MEJORA LA DISFUNCIÓN ENDOTELIAL Y EL REMODELADO DE LOS MICROVASOS DE RATONES APOE-/-

Perez-Ternero C, Rodriguez-Rodriguez R, Herrera MD, Alvarez de Sotomayor M

Atherosclerosis. 2016;250:15-22.

Las propiedades de resistencia y funcionales de las arterias mesentéricas desempeñan un papel clave en el mantenimiento de la homeostasis vascular. El objetivo de este trabajo fue la evaluación de la suplementación con el extracto enzimático de salvado de arroz (EESA) de la dieta de ratones deficientes en apolipoproteína E (ApoE-/-) sobre las propiedades funcionales, estructurales, miogénicas y de resistencia de las arterias mesentéricas.

Ratones ApoE-/- de 7 semanas de edad fueron alimentados durante 23 semanas con dieta baja (LFD) o alta en grasa y colesterol (HFD), suplementada o no al 1 o 5% con EESA. Como grupo control se emplearon ratones de genotipo salvaje (C57BL/6J) alimentados con dieta LFD. Las arterias mesentéricas se montaron en un miógrafo de presión para evaluar las propiedades estructurales, miogénicas y de resistencia. La reactividad vascular se evaluó en presencia de diferentes combinaciones de inhibidores de las síntesis de factores vasomotores de origen endotelial: L-NAME, indometacina, apamina y caribdotoxina.

Independientemente de la dieta administrada (LFD o HFD), los ratones ApoE-/- presentaron diferentes alteraciones estructurales y mecánicas que fueron mejoradas mediante la suplementación con EESA. Las arterias mesentéricas de los ratones C57BL/6J se caracterizaron por la participación de EDHF en la relajación vascular como consecuencia de la una mayor expresión de canales de potasio IK_{Ca}. Sin embargo, el papel del NO fue más relevante para los ratones ApoE-/-. La dieta HFD redujo la liberación de NO debido al incremento en la fosforilación de carácter inhibitorio de la enzima eNOS en Thr495. Este efecto fue completamente contrarrestado por la suplementación con EESA, mejorando la relajación a acetilcolina. La suplementación de la dieta HFD con EESA redujo los niveles de anión superóxido y de colágeno, evaluados mediante tinción con dihidroetidio y Sirius Red, respectivamente.

Con todo esto, podemos concluir que la suplementación de la dieta con EESA reduce el remodelado vascular y el estrés oxidativo de arterias mesentéricas.

Adicionalmente, el EESA incrementó la liberación de NO mediante la reducción de la fosforilación de eNOS en su residuo inhibitorio Thr495, protegiendo así la función endotelial.

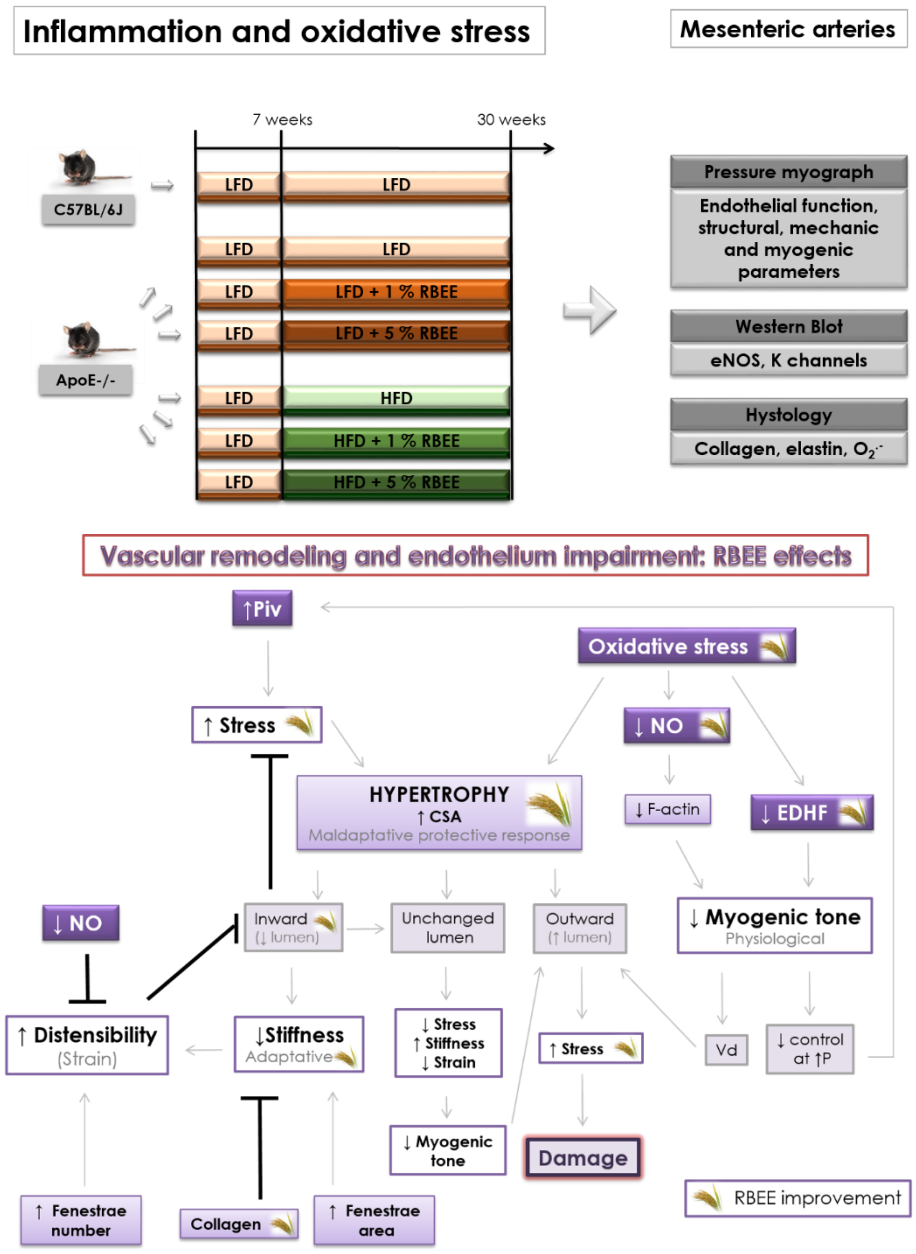

Figura 17: Diseño experimental y resumen de resultados.

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Diet supplementation with rice bran enzymatic extract restores endothelial impairment and wall remodelling of *ApoE*$^{-/-}$ mice microvessels

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ABSTRACT

Background and aims: Small mesenteric artery resistance and functionality are key factors for the maintenance of blood homeostasis. We attained to evaluate the effects of a rice bran enzymatic extract (RBEE) on structural, mechanic and myogenic alterations and endothelial dysfunction secondary to atherosclerosis disease.

Methods: Seven week-old *ApoE*$^{-/-}$ mice were fed on standard (ST) or high fat (HF) diets supplemented or not with 1 or 5% RBEE (w/w) for 23 weeks. Wild-type C57BL/6J mice fed on ST diet served as controls. Small mesenteric arteries were mounted in a pressure myograph in order to evaluate structural, mechanical and myogenic properties. Vascular reactivity was assessed in the presence of different combinations of inhibitors: L-NAME, indometacin, apamin and charybdotoxin.

Results: *ApoE*$^{-/-}$ mice fed on ST and HF diets showed different structural and mechanical alterations, alleviated by RBEE supplementation of ST and HF diets. C57BL/6J was characterized by increased expression of IK_{Ca} (199.3%, $p = 0.023$) and SK_{Ca} (133.2%, $p = 0.026$), resulting in higher EDHF participation ($p = 0.0001$). However, NO release was more relevant to *ApoE*$^{-/-}$ mice vasodilatation. HF diet reduced the amount of NO released due to 2-fold increase of eNOS phosphorylation in the inhibitory residue Thr495 ($p = 0.034$), which was fully counteracted by RBEE supplementation ($p = 0.028$), restoring ACh-induced vasodilatation ($p = 0.00006$). Dihydroethidium fluorescence of superoxide and picrosirius red staining of collagen were reduced by RBEE supplementation of HF diet by 76.91% ($p = 0.022$) and 65.87% ($p = 0.030$), respectively.

Conclusion: RBEE supplemented diet reduced vessel remodeling and oxidative stress. Moreover, RBEE supplemented diet increased NO release by downregulating p-eNOS^{Thr495}, thus, protecting the endothelial function.

1. Introduction

Atherosclerosis is characterized by the accumulation of fatty substances in the arterial wall. In conductance vessels, vessel remodelling as well as plaque adaptations to allow sufficient blood flow and rupture prevention have been well characterized. However, little is known regarding the events taking place in the small resistance vessels and its contribution to cardiovascular homeostasis in atherosclerosis [1]. Although resistance arteries are safe from plaque development, they play a key role in the control of blood flow and blood pressure. In this regard, a reciprocal control between conductance and resistance arteries can be found in the progression of atherosclerosis. Oxidative stress, high blood pressure and reduced vasorelaxant mediators such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) stimulate vascular cell proliferation and vessel remodelling (i.e. increased cross sectional area, CSA) aiming to reduce stress [2,3]. Nevertheless, outward and eutrophic hypertrophy remodelling results in increased stress and vessel damage on a long term basis [2,4].

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Moreover, reduction of NO and EDHF, the number of fenestrae and the relative elastine/collagen content can affect elasticity and thus myogenic tone [4–6].

Recently, interest has emerged in nutritional strategies to prevent and treat cardiovascular diseases [7]. Supplemented diets with the ability to change the structure, composition and function of the arteries may reduce the risk of developing atherosclerosis and thus, reduce mortality due to cardiovascular diseases. Rice bran is the outer layer of the rice grain and is rich in γ-oryzanol, phytosterols, tocols and ferulic acid which are known by their interesting lipid-lowering, antioxidant and anti-inflammatory potential [8,9]. Despite its potential for the treatment and prevention of human diseases such as obesity, hypertension, high cholesterol and other inflammation-related diseases [8–10], this layer is removed during rice milling and primarily used for animal feeding. The rice bran enzymatic extract (RBEE) used in this work has already shown its ability to restore vascular function in resistance arteries and in the aorta and to reduce oxidative stress in a rat model of metabolic syndrome [11–13] and to reduce plaque burden in *ApoE*$^{-/-}$ mice [14]. In addition to these *in vivo* actions, RBEE has technological advantages due to the extraction process with a mixture of endoproteases that avoids the characteristic rancidity and hydrophobicity of rice bran oil. This enzymatic extraction also increases some of the nutraceutical components compared to raw rice bran (Supplementary Table 1) [15].

The aim of this study was to characterize the effects of rice bran enzymatic extract chronic supplementation of chow and high fat diets on the structural, mechanical, myogenic and functional properties of small mesenteric arteries of *ApoE*$^{-/-}$ mice compared to wild type C57BL/6J.

2. Material and methods

2.1. Animals and diets

Six week-old male apolipoprotein E knockout (*ApoE*$^{-/-}$) mice on a C57BL/6J background and wild-type (C57BL/6J) mice were purchased from Charles River (Charles River Laboratories, L'Abresle, France). At seven weeks of age, *ApoE*$^{-/-}$ mice were randomly separated into six groups of treatment (n = 15). Three groups were fed on a standard chow diet (ST, Harlan Laboratories, Madison, USA) for 23 weeks: control standard diet (ST), 1% RBEE supplemented diet (ST1%) and 5% RBEE supplemented diet (ST5%). The last three groups were fed on a Western diet containing 0.2% (% kcal) cholesterol and 42% (% kcal) fat (HF, Harlan Laboratories, Madison, USA) for 23 weeks: control high fat diet (HF), 1% RBEE supplemented diet (HF1%) and 5% RBEE supplemented diet (HF1%). As non-atherosclerotic control, wild type C57BL/6J mice were kept on normal chow diet for the same period. All experimental procedures were conducted in accordance with the guidelines of the European Union for ethical management of animals and were approved by the Committee of Ethical Experimentation of the University of Seville (Spain) (AGL2013-47791-P).

2.2. Rice bran enzymatic extract

The water-soluble syrup RBEE, generously provided by Dr. Parrado research group, was prepared as previously described [15]. Briefly, the process involves enzymatic hydrolysis in a bioreactor pH (pH 8) and temperature (60 °C) controlled, using an endoprotease mixture, which lead to endogenous lipase inactivation. Chemical characterisation of the extract used in this work resulted as follows: peptides and free amino acids are the major component (38.1%), whose interaction with fat components (30%) permits its solubilisation. An increase in other nutraceutical components in the extract was described as listed: γ-oryzanol (8950 mg/kg), phytosterols (3553 mg/kg), tocotrienols (170 mg/kg) and tocopherols (93.4 mg/kg). Full description of the experimental diets, raw rice bran and RBEE can be found in Supplementary Table 1.

2.3. Artery isolation and pressure myography

Vascular function and structural, mechanical and myogenic parameters were studied by pressure myography (111P, Danish Myo Technology, Aarhus, Denmark) as described previously [16]. Second-order-branches of cleaned small mesenteric arteries were tired onto two glass microcannulas and left to equilibrate for 30 min at 37 °C and 70 mmHg in modified Krebs-Henseleit solution (KHS) of the following composition (mmol/L): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.18 and glucose 11. Then, pressure-diameter curves (5–120 mmHg) obtained with internal and external diameters (D_{iCa} and D_{eCa}) were measured for 3 min at each intraluminal pressure. Finally, the artery was left to equilibrate in calcium-free KHS (KHS without $CaCl_2$ and containing 10 mmol/L EGTA) and afterwards a second pressure-diameter curve was performed.

2.3.1. Calculation of structural, mechanical and myogenic parameters

The structural properties, wall thickness (wt) and wall cross sectional area (CSA) and the mechanical properties, circumferential stress (σ) and circumferential strain (ε), were calculated from observed changes in the vessel diameter relative to the increase in intraluminal pressure in passive conditions as previously described [16]. In order to determine arterial stiffness, the stress-strain curve was used to obtain the elastic modulus and the index of stiffness (β) was calculated as described elsewhere [16]. Myogenic response percentages were finally calculated comparing internal diameter in active (1.8 mmol/L Ca^{2+} KHS) and passive (0 Ca^{2+} KHS) conditions [16].

2.3.2. Assessment of vascular functionality

Following equilibration, arteries were constricted with 10 μmol/L Phe and cumulative concentration-response curves to ACh (0.001–30 μmol/L) were constructed. Then, another two curves were performed after 30 min incubation with each of the following inhibitors: the NOS inhibitor L-NAME (300 μmol/L) alone or in combination with the non-selective cyclooxygenase inhibitor indometacin (10 μmol/L) and the small-conductance calcium-activated potassium (SK_{Ca}) channel blocker apamin (100 nmol/L) in combination with the blocker of both SK_{Ca} and intermediate-conductance calcium-activated potassium channels (IK_{Ca}) charybdotoxin (50 nmol/L), which mediate EDHF response. Vasodilatation was expressed as a percentage of the previous tone generated by Phe. Then, the area under the curve (AUC) of NO and EDHF was calculated as the difference between the area under the curve of the concentration response curve to ACh alone and in presence of L-NAME and as the difference between the area under the curve of the concentration response curve to ACh in presence of L-NAME and indometacin and in presence of L-NAME, indometacin, apamin and charybdotoxin, respectively.

2.4. Measurement of vascular O_2^- production, elastin and collagen content

To evaluate the production of superoxide anion (O_2^-) *in situ*, we used the oxidative fluorescent dye dihydroethidium (DHE). Briefly, 14 μm thick frozen arterial sections were placed in gelatine coated slides and were incubated with fresh buffer containing 2 μmol/L DHE. Ethidium bromide fluorescence and elastin autofluorescence

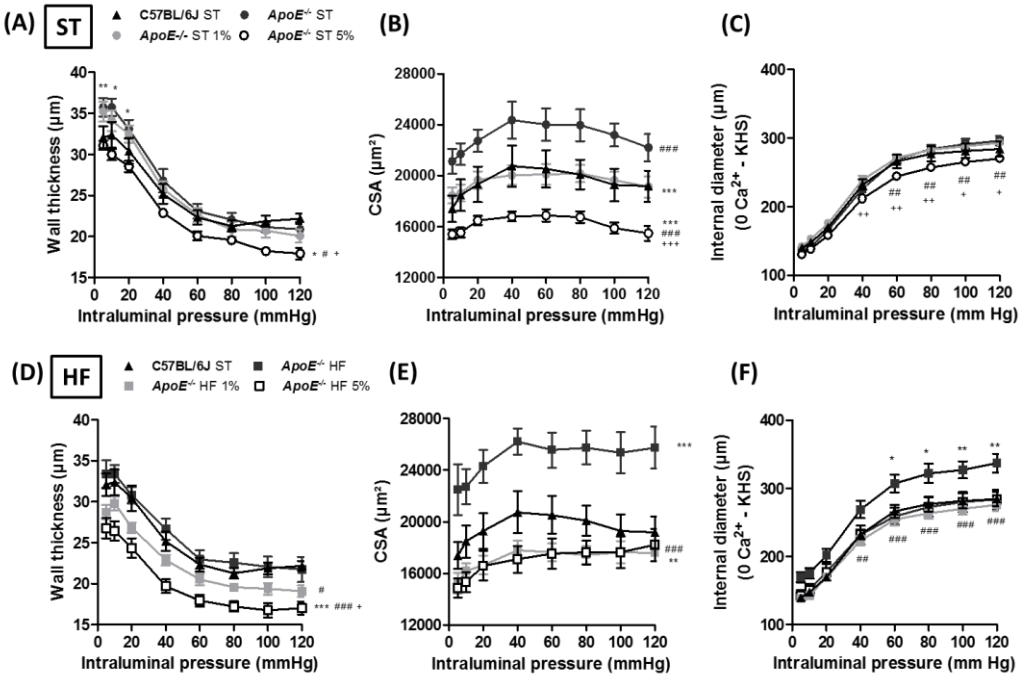

Fig. 1. Structural properties of fully relaxed small mesenteric arteries. C57BL/6J and $ApoE^{-/-}$ mice were fed on standard (ST, upper panels) or high fat (HF, lower panels) supplemented or not diets with 1 or 5% RBEE. (**A** and **D**) Wall thickness-intraluminal pressure. (**B** and **E**) Cross-sectional area (CSA)-intraluminal pressure. (**C** and **F**) Internal passive diameter-intraluminal pressure. Values are mean ± SEM (n = 9–15 mice, one vessel studied per animal). Significant differences are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* C57BL/6J, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* $ApoE^{-/-}$ ST or HF and $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ *vs* ST1% or HF1%, and were determined using ANOVA followed by Bonferroni's test for multiple comparisons.

were visualized by confocal laser scanning (40x objective; Zeiss LSM 7 Duo; Zeiss, Oekochen, Germany). Collagen was determined in 10 µm thick frozen arterial sections with stained picrosirius red and was visualized using polarized microscopy (40x objective, Olympus BX61). Quantitative analysis was performed with ImageJ software 1.47v (NIH, USA) and was normalized with the tissue area observed.

2.5. Protein expression

A protein extraction kit (Nuclear extract kit, Active Motif, Belgium) was used according the manufacturer's instructions for whole protein extraction from small mesenteric arteries. Equal amounts of protein were resolved on a 10% SDS-polyacrylamide gel, transferred to PVDF membranes and incubated at 4 °C overnight with one of the following primary antibodies: anti-eNOS, anti-p-$eNOS^{Ser1177}$, anti-p-$eNOS^{Thr495}$ (1:400, Cell Signalling Technology, Beverly, USA), anti-IK_{Ca}, anti-SK_{Ca} (1:800, Santa Cruz Biotechnology, Heidelberg, Germany), anti-$p22^{phox}$ (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) or anti-Nox-1 (1:1000, Novus Biologicals, Cambridge, UK). Antibody binding was visualized using an enhanced chemiluminescence detection system (Fujifilm LAS-3000 Imager; Stamford, USA) and densitometric analysis was performed with the ImageJ v1.45 software (NIH, USA) using β-actin as internal control to verify equal protein loading.

2.6. Statistical analysis

Results are expressed as mean ± SEM of the number of experiments. One- or two-way ANOVA analysis was performed, followed by Bonferroni's test for multiple comparisons and statistical comparisons were evaluated using the GraphPad Prism Software v5.01 (San Diego, USA). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Effects of diets and RBEE on structural properties

Vessel diameters were measured over the pressure range from 5 to 120 mmHg to evaluate the structural parameters. $ApoE^{-/-}$ fed on ST and HF maintained wall thickness (wt) (Fig. 1 A and D), but had bigger CSA than C57BL/6J (Fig. 1 B and E, $p < 0.001$), showing eutrophic hypertrophy in the case of ST and outward hypertrophy in the case of HF as a result of increased internal diameter in passive conditions (Fig. 1F, $p < 0.05$). Supplementation of ST diet with 5% RBEE resulted in reduced wt, CSA and internal diameter (Fig. 1 A, B and C, wt: $p < 0.05$, CSA: $p < 0.001$ and internal diameter $p < 0.05$ *vs* ST and C57BL/6J). Under HF diet, supplementation with both 1 and 5% RBEE decreased wt (Fig. 1D, HF1% $p < 0.05$ *vs* C57BL/6J; HF5%: $p < 0.001$ *vs* C57BL/6J and HF), CSA (Fig. 1E, $p < 0.001$ and $p < 0.01$ *vs* HF and C57BL/6J) and internal diameter (Fig. 1F, $p < 0.001$). Wall to lumen ratio was not altered in this study (data not shown). To find out whether 1 and 5% doses of RBEE supplemented diet had different effects depending on the diet, further comparisons were performed (Supplementary Figs. 1 and 2). The effects of RBEE supplementation of ST and HF diets were comparable regarding wt, which was bigger for both ST1% (Supplementary Fig. 1A) and ST5% (Supplementary Fig. 2A) compared to HF. However, only the dose of 1% in HF reduced CSA (Supplementary Fig. 2B), while internal diameters in active conditions were not affected by the different doses of RBEE (Supplementary Fig. 1C and 2C).

3.2. Effects of diets and RBEE on myogenic response

Fig. 2 illustrates the relation of pressure and internal diameter as intraluminal pressure increased stepwise from 5 to 120 mmHg in active conditions (1.8 mmol/L Ca^{2+} KHS). Internal diameter of small mesenteric arteries from $ApoE^{-/-}$ under ST (A) and HF (B) diets

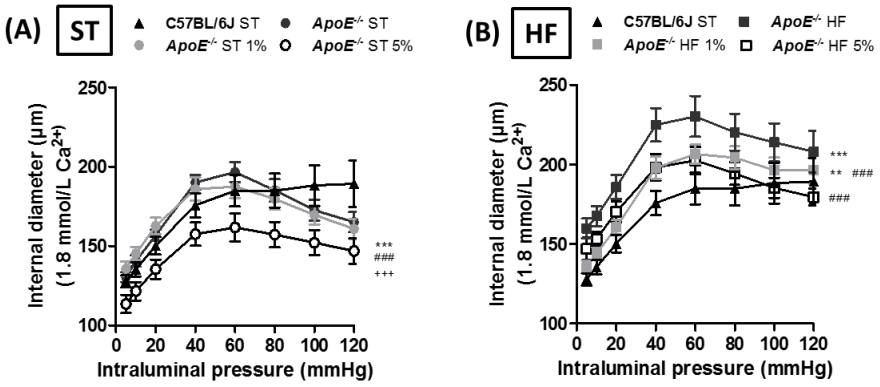

Fig. 2. Myogenic properties of small mesenteric arteries. (**A**) Internal active diameter-intraluminal pressure of C57BL/6J and $ApoE^{-/-}$ mice fed on standard (ST) supplemented or not diet with 1 or 5% RBEE. (**B**) Internal active diameter-intraluminal pressure of C57BL/6J and $ApoE^{-/-}$ mice fed on high fat (HF) supplemented or not diet with 1 or 5% RBEE. Values are mean ± SEM (n = 10–15, mice, one vessel studied per animal). Significant differences are indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* C57BL/6J, $^{###}p < 0.001$ *vs* $ApoE^{-/-}$ ST or HF and $^{+++}p < 0.001$ *vs* ST1%, and were determined using ANOVA followed by Bonferroni's test for multiple comparisons.

increased as the intraluminal pressure raised and, in contrast to C57BL/6J, significant hysteresis was detected from 60 to 120 mmHg. 5% RBEE ST diet supplementation markedly reduced internal diameter compared to ST fed mice (Fig. 2A, $p < 0.001$). Similarly, supplementation of HF diet with 1 and 5% RBEE reduced significantly lumen diameter compared to HF mice (Fig. 2B, $p < 0.001$). The pressure-dependent myogenic tone generated in active relative to passive conditions by ST and HF fed mice remained unchanged compared to C57BL/6J mice and was not affected by RBEE supplementation (Supplementary 3 A and B).

3.3. Effects of diets and RBEE on endothelial function

Endothelium-dependent ACh-induced relaxation was significantly lower in all $ApoE^{-/-}$ groups compared to C57BL/6J (Fig. 3 A and B). Although pEC_{50} remained unchanged, the maximum response to ACh was attenuated in $ApoE^{-/-}$ mice compared to C57BL/6J. This effect was especially remarkable in arteries of HF fed mice (E_{max}: 121.2 ± 13.96, 64.38 ± 7.48 and 48.23 ± 7.05 for C57BL/6J, ST and HF, respectively; $p < 0.001$). RBEE supplemented HF diets restored partially the ACh response (E_{max}: 84.52 ± 9.56 and 93.12 ± 14.61 for HF1% and HF1%, respectively; $p < 0.001$).

To further investigate the different endothelial-derived components involved in that response, additional curves were made in presence of several inhibitors for NO and EDHF release and AUC were calculated. Inhibition of NOS did not change C57BL/6J response to ACh (E_{max}: 122.4 ± 22.37). However, $ApoE^{-/-}$ mice experienced a marked reduction in the endothelium-dependent relaxation (E_{max}: 33.37 ± 5.10 and 34.33 ± 6.92 for ST and HF, respectively; $p < 0.001$). NO contribution to ACh-induced vasorelaxation in $ApoE^{-/-}$ mice was reduced by HF ($p < 0.001$) and was completely restored by 1 and 5% RBEE supplementation (Fig. 3C, $p < 0.05$). Interestingly, the contribution of EDHF to vasorelaxation in all $ApoE^{-/-}$ mice was markedly lower compared to C57BL/6J regardless of the diet (Fig. 3D, $p < 0.001$).

3.4. Effects of diets and RBEE on eNOS phosphorylation, IK_{Ca} and SK_{Ca} protein expression

Western blot analyses showed increased eNOS expression in the small mesenteric arteries from ST fed $ApoE^{-/-}$ mice compared to C57BL/6J and $ApoE^{-/-}$ HF fed (Fig. 4A, $p < 0.01$). When the phosphorylated forms of eNOS were measured, higher expression of p-eNOS at Ser1177 was found in ST fed $ApoE^{-/-}$ mice (Fig. 4B, $p < 0.05$), probably related to the higher expression of total eNOS and not to a higher activation since the p-eNOS to total eNOS ratio was not changed (Supplementary 4A). HF diet or RBEE supplemented diets did not change phosphorylation at Ser1177. On the other hand, $ApoE^{-/-}$ mice fed on HF diet showed increased inhibition, measured by phosphorylation at Thr495 ($p < 0.05$) and supplementation with 5% RBEE counteracted such inhibition (Fig. 4C, $p < 0.05$), which was confirmed by the ratio of phosphorylated eNOS at Thr495 to total eNOS (Supplementary 4B, $p < 0.05$). Small mesenteric arteries from all $ApoE^{-/-}$ mice showed lower expression of both SK_{Ca} and IK_{Ca} compared to C57BL/6J (Fig. 4 D and E). $ApoE^{-/-}$ mice fed on HF diet showed lower expression of SK_{Ca} compared to $ApoE^{-/-}$ mice fed on ST diet (Fig. 4E, $p < 0.05$). RBEE supplemented diet did not modify the expression of SK_{Ca} and IK_{Ca} channels.

3.5. Effects of diets and RBEE on collagen and elastin content and mechanical properties

Collagen content measurement by picrosirius red staining showed no difference between small mesenteric arteries from $ApoE^{-/-}$ and C57BL/6J mice fed on ST diets. However, HF diet increased the deposition of collagen (Fig. 5A and C; $p < 0.05$ and $p < 0.001$ *vs* C57BL/6J and ST fed $ApoE^{-/-}$, respectively). Such increase was completely counteracted by 5% RBEE diet supplementation (Fig. 5C, $p < 0.01$). Estimations of total elastin content by confocal microscopy showed no difference between small mesenteric arteries from mice fed on ST but higher elastin content in vessels from $ApoE^{-/-}$ mice fed on HF diets (Fig. 5D).

Stiffness is related to collagen and elastin content. To further investigate stiffness, analysis of the stress-strain curve was performed (Supplementary 5 A and B) and β value was calculated. $ApoE^{-/-}$ ST group showed reduced stiffness (Fig. 5E, β: 3.80 ± 0.15) compared to C57BL/6J (β: 4.47 ± 0.20, $p < 0.05$) which was counteracted by 1 and 5% diet supplementation (Fig. 5E, β: 5.18 ± 0.25 and 4.34 ± 0.17 for ST1% and ST5%, respectively). However, HF group experimented increased stiffness compared to C57BL/6J (Fig. 5E, β: 5.18 ± 0.25, $p < 0.05$) that was prevented by 5% RBEE supplementation as evidenced by the rightward shift of the stress-strain relationship (β: 4.39 ± 0.17, $p < 0.05$).

3.6. Effects of diets and RBEE on vascular O_2^- production

Superoxide levels, assessed by the fluorescence of DHE, were

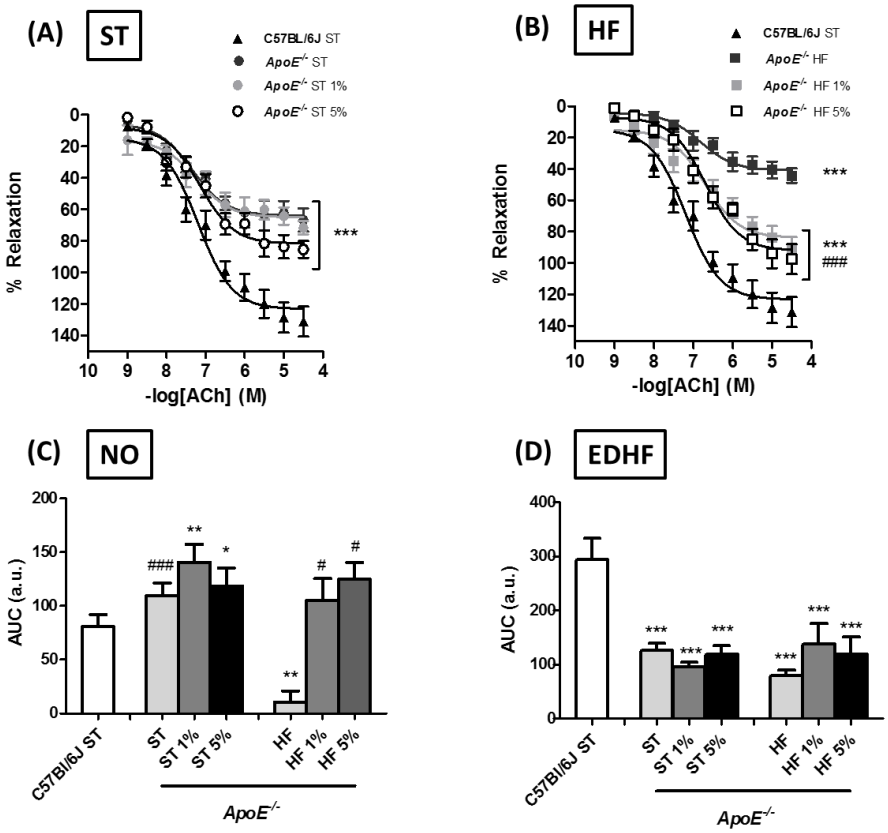

Fig. 3. Functional properties of small mesenteric arteries. (**A**) Concentration-response curves of C57BL/6J and *ApoE*$^{-/-}$ mice fed on supplemented or not standard diet (ST) with 1 or 5% RBEE. (**B**) Concentration-response curves of C57BL/6J and *ApoE*$^{-/-}$ mice fed on high fat (HF) supplemented or not diet with 1 or 5% RBEE. (**C**) Area under the curve (AUC) of nitric oxide (NO) represented as the difference between the area under the curve of the concentration response curve to ACh alone and in the presence of the NO synthase inhibitor (L-NAME, 300 μmol/L). (**D**) AUC of endothelium-dependent hyperpolarizing factor (EDHF) represented as the difference between the area under the curve of the concentration response curve to ACh in the presence of L-NAME and with indometacin (10 μmol/L) and in presence of L-NAME, indometacin, apamin and charybdotoxin. Values are mean ± SEM. (n = 6–8 mice, one vessel studied per animal). Significant differences are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* C57BL/6J and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs* HF, and were determined using ANOVA followed by Bonferroni's test for multiple comparisons.

significantly higher in *ApoE*$^{-/-}$ mice fed both on ST and HF diets compared to C57BL/6J mice (Fig. 5F, $p < 0.05$ and $p < 0.01$ for ST and HF, respectively). The fluorescence intensity of the HF group was significantly reduced in vessels of mice receiving with 1% RBEE ($p < 0.05$), but not in the 5% group. To further investigate RBEE effects on $O_2^{\cdot-}$ production, two subunits of the NADPH oxidase enzyme complex were measured. While ST diet did not change the expression of Nox-1 subunit with ST diet, this protein expression was upregulated by HF diet (Fig. 5 H, $p < 0.05$). 1% supplements of HF diet counteracted such effect ($p < 0.05$). The expression of $p22^{phox}$ was not affected in *ApoE*$^{-/-}$ mice and unchanged by the introduction of RBEE in the diet.

4. Discussion

The present study provides new insights into the effects of RBEE supplementation of ST and HF diets regarding the endothelium-dependent relaxation and structural and mechanical alterations of pressurized small mesenteric arteries of *ApoE*$^{-/-}$ mice compared to wild type C57BL/6J mice. NO release was lower in *ApoE*$^{-/-}$ compared to C57BL/6J and was further impaired by HF diet through promotion of eNOS inhibition. Nonetheless, NO was identified as the main responsible for endothelium-dependent vasorelaxation in *ApoE*$^{-/-}$ mice. RBEE diet supplementation prevented eNOS inhibition. In contrast, EDHF-derived vasorelaxation was more relevant to C57BL/6J mice. Significant hypertrophy, as shown by increased CSA of *ApoE*$^{-/-}$ mice fed on ST and HF diets, was alleviated by RBEE supplementation. Moreover, enhanced stiffness, confirmed by increased collagen content and β value, was also reduced by the dietary supplementation with RBEE, showing for the first time how a diet supplement can preserve vascular function and prevent microvascular remodelling.

The main function of resistance arteries is to control blood flow so that an ideal pressure-flow relationship is maintained. Structural changes and endothelium impairment may undergo as a consequence of several cardiovascular diseases or aging [16,17], leading to remodelling and mechanical and myogenic adaptations [3,18]. Reduced stiffness and increased distensibility are the adaptive mechanisms aiming to compensate vascular stress and potential lumen narrowing arising from increased CSA [3]. Here, *ApoE*$^{-/-}$ fed on HF diet showed outward hypertrophy which was characterized by increased CSA and lumen diameter. Nevertheless, outward hypertrophy failed to compensate vascular stress, revealing an advanced remodelling state, which reinforced by the increased stiffness is a predictor of cardiovascular disease [19]. In contrast, *ApoE*$^{-/-}$ fed on ST, despite having increased CSA, were still able to maintain stress within control values. In agreement with our findings, increased distensibility and reduced vascular stiffness were found in microvessels of experimental models of obesity, ischemia, liver hypertension and atherosclerosis [4,6,16]. Treatment with RBEE was able to compensate this remodelling, both in ST and HF diets. HF-treated mice receiving RBBE showed reduced stiffness

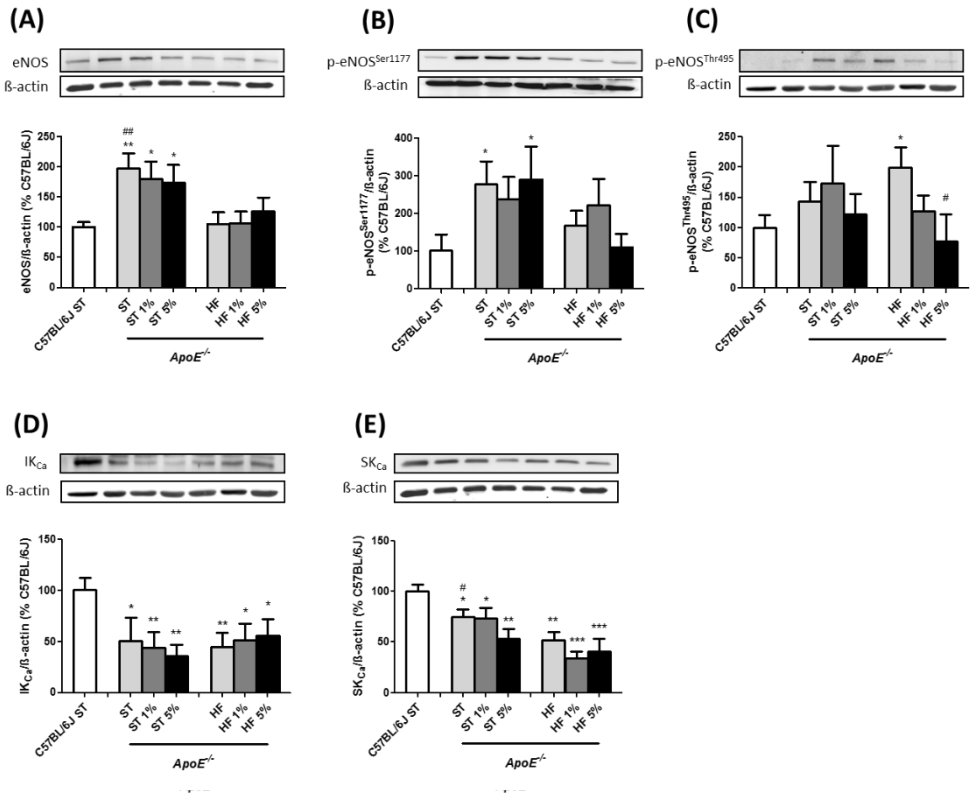

Fig. 4. Expression of endothelium-dependent relaxation proteins. C57BL/6J and *ApoE*$^{-/-}$ mice were fed on standard (ST) or high fat (HF) supplemented or not diets with 1 or 5% RBEE. (**A**) eNOS. (**B**) p-eNOS^{Ser1177}, (**C**) p-eNOS^{Thr495}, (**D**) IK_{Ca}. (**E**) SK_{Ca}. Values are mean ± SEM (n = 4–9 mice, one vessel studied per animal). Significant differences are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* C57BL/6J, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs* HF, and were determined using ANOVA followed by Bonferroni's test for multiple comparisons.

and associated increased distensibility, resulting in reduced stress while RBEE supplement of either ST or HF diet induced a reduction of CSA. We hypothesise that this amelioration was achieved not only by direct effects in the mesenteric vascular bed due to reduced oxidative stress and collagen deposition, but also as a result of lipid profile amelioration and plaque burden reduction as previously described [14]. These changes in the structure leading to vessel remodelling were especially noteworthy in HF 5% group where wt was thinner and the CSA was considerably reduced possibly related to the reduction of collagen deposition observed in this group.

The elastic and myogenic properties of the vessels mainly depend on collagen (more rigid) and elastin (more flexible) burden [20,21]. Unlike ST, HF diet increased elastin content, without any differences induced by the RBEE treatment. Also, HF diet-fed-mice had increased content in collagen resulting in unchanged myogenic properties but had stiffness as evidenced by the increased β value and the moderate rightward shift of the stress-strain curve, which were alleviated by the RBEE treatment. We have previously reported increased collagen content and stiffness trend in mesenteric arteries of *ApoE*$^{-/-}$ mice fed on HF diet [16]. Others have reported increased content of elastin and collagen in the aortas of atherosclerotic rabbits [22], and increased elastic modulus in the aortas of *ApoE*$^{-/-}$ mice which the authors related to greater collagen recruitment at lower pressures [23].

The loss of vascular functionality is a hallmark in the onset of several cardiovascular diseases such as hypertension, diabetes and atherosclerosis [24]. Atherosclerotic plaque development leads to focal endothelial dysfunction and wall remodelling. In contrast, resistance arteries are safe from plaque burden, but as shown in this work, not from developing endothelial dysfunction. In this present study, we found NO to be the main responsible for endothelial-dependent vasodilatation in *ApoE*$^{-/-}$ mice and that HF diet has a deleterious effect in NO release by eNOS. Phosphorylation at Ser1177 and Ser633 enhances eNOS activity, whereas phosphorylation of Thr495 and Ser116 inhibits eNOS activity decreasing vasodilatation [25]. In this work it is shown for the first time that *ApoE*$^{-/-}$ mice under standard diet upregulated eNOS expression in small arteries compared to wild type C57BL/6J and *ApoE*$^{-/-}$ mice fed on HF diet. This upregulation of eNOS occurred without changing eNOS phosphorylation in Ser1177. On the other hand, HF induced phosphorylation at the inhibitory site Thr495 and consequently reduced NO release and impaired vasodilatation were observed. Supplementation of HF diet with RBEE restored eNOS activity by reducing phosphorylation at Thr495. According to this result, others have reported HF-mediated impairment of the mesenteric vascular function [26] while we previously found NO mediated relaxation improvement by RBEE diet supplementation in microvessels from obese Zucker rats [24]. Moreover, NO bioavailability may be reduced by eNOS uncoupling leading to O_2^- production [27], which is also enhanced by NADPH oxidase activation [28]. HF diet fed mice evidenced oxidative state characteristic of atherosclerosis disease through increased O_2^- production, as revealed by *in situ* O_2^- measurement and increased Nox-1 expression. 1% RBEE supplements reduced O_2^- production due to the downregulation of NADPH oxidase subunit expression as we previously reported in small mesenteric arteries of obese Zucker rats [13] owing to high content of antioxidant compounds such as tocols or γ-oryzanol in RBEE. However, no protective effect on oxidative stress was observed at the higher dose provided by 5% RBEE supplements. This fact could be related to the dual effect of tocotrienols

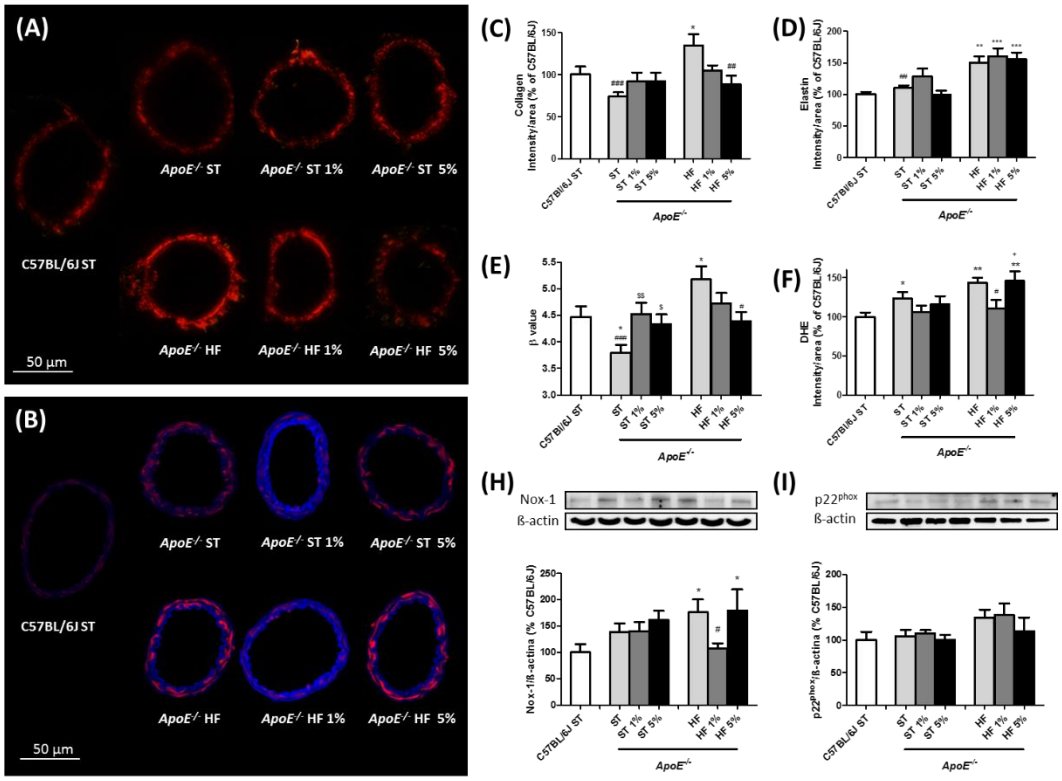

Fig. 5. Oxidative stress of small mesenteric arteries. C57BL/6J and *ApoE*$^{-/-}$ mice were fed on standard (ST) or high fat (HF) diets supplemented or not with 1 or 5% RBEE. (**A**) Collagen staining with picrosirius red. (**B**) DHE fluorescence and elastin autofluorescence. (**C**) Intensity quantification of collagen staining. (**D**) Intensity quantification of elastin autofluorescence (**E**) β values (arterial wall stiffness). (**F**) Intensity quantification of DHE fluorescence. Representative images and quantification of NADPH oxidase subunits: (**G**) Nox-1 (**H**) p22^{phox}. Values are mean ± SEM (n = 5–8 mice, two independent vessels studied per animal). Significant differences are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* C57BL/6J, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* HF, $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ *vs* ST and $^{+}p < 0.05$ *vs* HF1%, and were determined using ANOVA followed by Bonferroni's test for multiple comparisons.

which can be converted to tocopherols *in vivo* and while at low dose exert antioxidant activities, at high dose may become prooxidant. Therefore, higher supply of tocols by HF 5% diet may explain the loss of protection compared to HF 1% [29]. Nevertheless, RBEE endothelial protection should be attributed to actions related to eNOS activity and not directly to the antioxidant action of its components since both 1 and 5% RBEE supplements counteracted HF deleterious effect on ACh-dependent relaxation but only 1% reduced O_2^- release by NADPH oxidase. Several authors reported that the rice bran component ferulic acid, which is also part of the γ-oryzanol structure, as well as rice bran peptides may protect the endothelial function by inducing eNOS activity and expression and reducing inflammatory markers through inhibition of NF-κβ pathways [30–32].

Endothelial-derived vasodilatation not only relies on NO, but also on prostacyclin and on EDHF. Although the study of cyclooxygenase-derived mediators in our model did not revealed their implication in endothelium derived vasorelaxation, the inhibition of prostacyclin production allowed us to measure the EDHF role in endothelium-dependent relaxation. EDHF diffuses to smooth muscle cells and activates K^+ channels, inducing smooth muscle hyperpolarization and relaxation [33]. Interestingly, we found that EDHF participation was considerably greater in wild type C57BL/6J mice compared to *ApoE*$^{-/-}$ and thence an increased relaxation was observed. EDHF participation was confirmed by upregulation of potassium channels, while *ApoE*$^{-/-}$ showed lower levels. Conversely, Beleznai T. and co-workers found comparable EDHF participation in small mesenteric arteries of 40–44 week-old *ApoE*$^{-/-}$ mice fed on ST diet compared to wild type [34].

In conclusion, this work describes RBEE diet supplementation beneficial effects on the endothelium-dependent relaxation of *ApoE*$^{-/-}$ mice, revealing NO release by eNOS and oxidative stress amelioration as the main mechanisms. Moreover, the endothelium-dependent relaxation components were characterized, revealing EDHF as the main contributor to ACh-induced response in wild type C57BL/6J mice while NO was for *ApoE*$^{-/-}$ mice. In addition, RBEE treatment was able to ameliorate remodelling in *ApoE*$^{-/-}$ mice despite the type of diet, arising as an interesting nutraceutical ingredient in the control of atherosclerotic-related disorders.

Author contribution

All of the authors listed above have contributed to the work and have read, participated in the writing and agreed upon the submitted version of the manuscript.

The document is original and it has not been published or submitted elsewhere.

Ethical approval

The animal studies performed throughout the investigation have been reviewed by the appropriate Ethics Committees.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.023.

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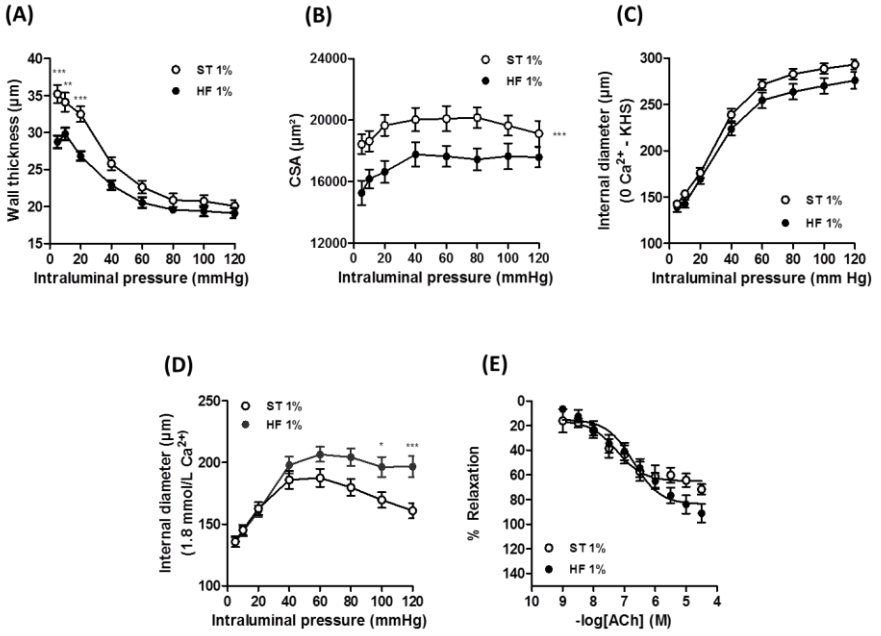
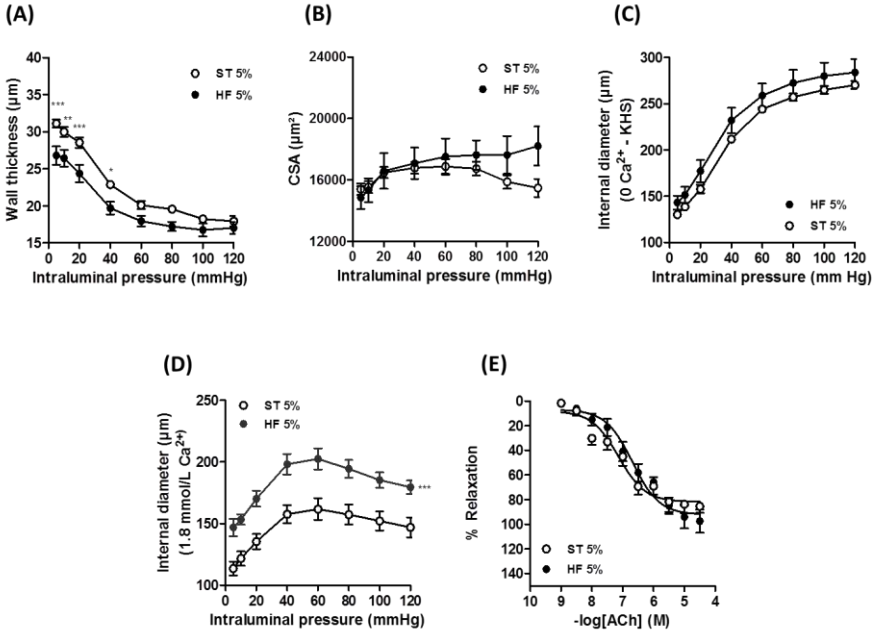
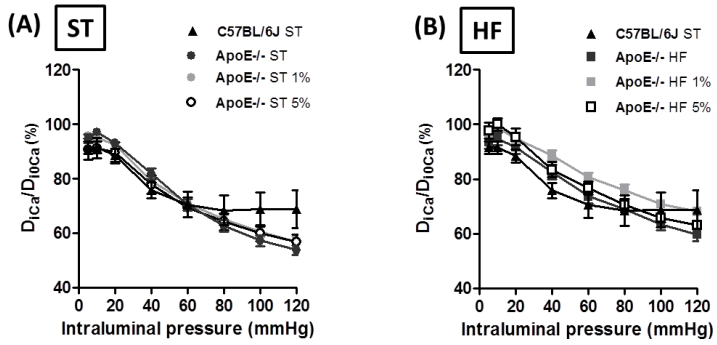


Supplementary 1. Effects of 1% RBEE supplementation of HF and ST diets. (**A**) Wall thickness-intraluminal pressure. (**B**) Cross-sectional area (CSA)-intraluminal pressure. (**C**) Internal passive diameter-intraluminal pressure. (**D**) Internal active diameter-intraluminal pressure. (**E**) Concentration-response curves to acetylcholine (ACh). Values are mean ± SEM. Significant differences are indicated by *p < 0.05, **p < 0.01 and ***p < 0.001, and were determined using

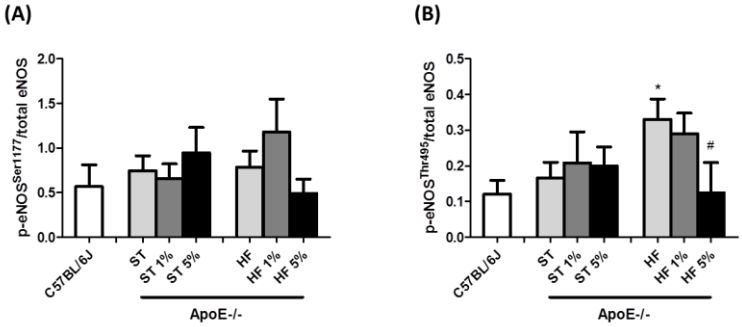


ANOVA followed by Bonferroni's test for multiple comparisons.

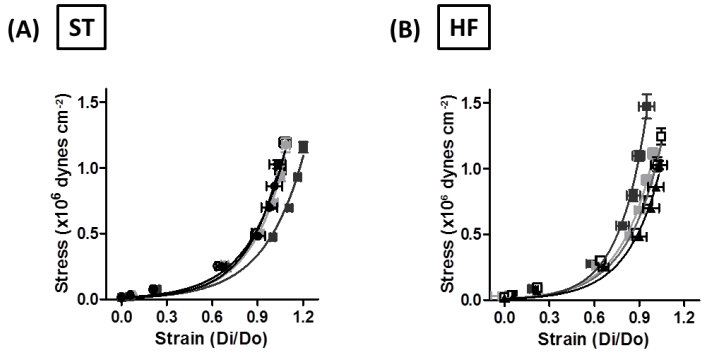
Supplementary 2. Effects of 5% RBEE supplementation of HF and ST diets. (**A**) Wall thickness-intraluminal pressure. (**B**) Cross-sectional area (CSA)-intraluminal pressure. (**C**) Internal passive diameter-intraluminal pressure. (**D**) Internal active diameter-intraluminal pressure. (**E**) Concentration-response curves to acetylcholine (ACh). Values are mean ± SEM. Significant differences are indicated by *p < 0.05, **p < 0.01 and ***p < 0.001, and were determined using ANOVA followed by Bonferroni's test for multiple comparisons.



Supplementary 3. Myogenic properties of small mesenteric arteries. Myogenic response (D_{iCa}/D_{i0Ca})-intraluminal pressure from C57BL/6J and *ApoE*$^{-/-}$ mice fed on (**A**) standard (ST) or (**B**) high fat (HF) supplemented or not diets with 1 or 5% RBEE. D_{iCa} and D_{i0Ca} internal diameter measured in active conditions (1.8 mmol/L Ca^{2+} KHS) and passive conditions (0Ca^{2+} KHS), respectively. Values are mean ± SEM (n = 5–9 mice, two independent vessels studied per animal).



Supplementary 4. Western blot analysis of posttranscriptional eNOS modifications by phosphorylation at Ser1177 and Thr495 residues. (**A**) Ratio of p-eNOS^{Ser1177} to total eNOS (**B**) Ratio p-eNOS^{Thr495} to total eNOS. Values are mean ± SEM (n = 4–6 mice, one vessel studied per animal). Significant differences are indicated by *p < 0.05 *vs* C57BL/6J, #p < 0.05 and #p < 0.01 *vs* HF, and were determined using ANOVA followed by Bonferroni's test for multiple comparisons.



Supplementary 5. Mechanical properties of fully relaxed small mesenteric arteries. Stress-strain (Di/Do) of (**A**) Standard diet (ST) or (**B**) high fat supplemented or not diet with 1 or 5% RBEE. Values are mean ± SEM (n = 10–15 mice, one vessel studied per animal).

CAPÍTULO IV

A veces Dios cambia las circunstancias porque Él está usando las circunstancias para cambiarte a ti.

LA INFLAMACIÓN Y EL ESTRÉS OXIDATIVO DERIVADOS DEL DESARROLLO DE ATEROSCLEROSIS MEJORAN POR EL TRATAMIENTO CON EL EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ

Perez-Ternero C, Bermudez Pulgarín B, Alvarez de Sotomayor M, Herrera MD

Journal of Functional Foods. 2016;5:1673-1683.

El salvado de arroz se ha considerado como un interesante suplemento nutricional para la prevención de enfermedades cardiovasculares dadas sus conocidas propiedades antioxidantes y antiinflamatorias. El objetivo de este trabajo es profundizar en los mecanismos antioxidantes y antiinflamatorios que contribuyen a explicar las acciones antiateroscleróticas del EESA, intentando dilucidar cuáles de su principios activos son responsables de estos efectos.

Ratones deficientes en apolipoproteína E (ApoE-/-) fueron alimentados con una dieta alta en grasa y colesterol suplementada o no al 1 o 5% con el extracto enzimático de salvado de arroz (EESA) durante 23 semanas. Paralelamente, monocitos humanos fueron inducidos con LPS (100 ng/ml) en presencia de EESA, ácido ferúlico (FA) (20 µg/ml) o γ-oryzanol (γ-Ory) (20 µg/ml).

La suplementación de la dieta con EESA redujo la activación de NF-κβ y la expresión de TNF-α, iNOS y COX-2 en la aorta y de los metabolitos del NO en el suero. Adicionalmente, se redujo la expresión de las subunidades de NADPH oxidasa Nox-1 y $p47^{phox}$, independientemente de la dosis de EESA, y $p22^{phox}$ en la dosis de 1% EESA. Como consecuencia, disminuyó la deposición de LDL oxidadas en la aorta y la producción de anión superóxido, medido mediante fluorescencia de DHE. En monocitos humanos, la incubación con EESA, FA y γ-Ory redujo el perfil proinflamatorio (CD14++ CD16+) e incrementó el de monocitos no clásicos (CD14- CD16++). Además, se observó un viraje hacia macrófagos M2, lo que redujo los niveles de IL-6 y TNF-α.

Con todo esto, podemos concluir que el consumo regular de EESA junto con una dieta alta en grasa reduce el estrés oxidativo y la inflamación derivados del proceso aterosclerótico, mostrando su interés como suplemento nutricional. Como principal responsable de los efectos antiinflamatorios observados se identificó al ácido ferúlico, presente en el EESA libre y como parte estructural de la molécula de γ-oryzanol.

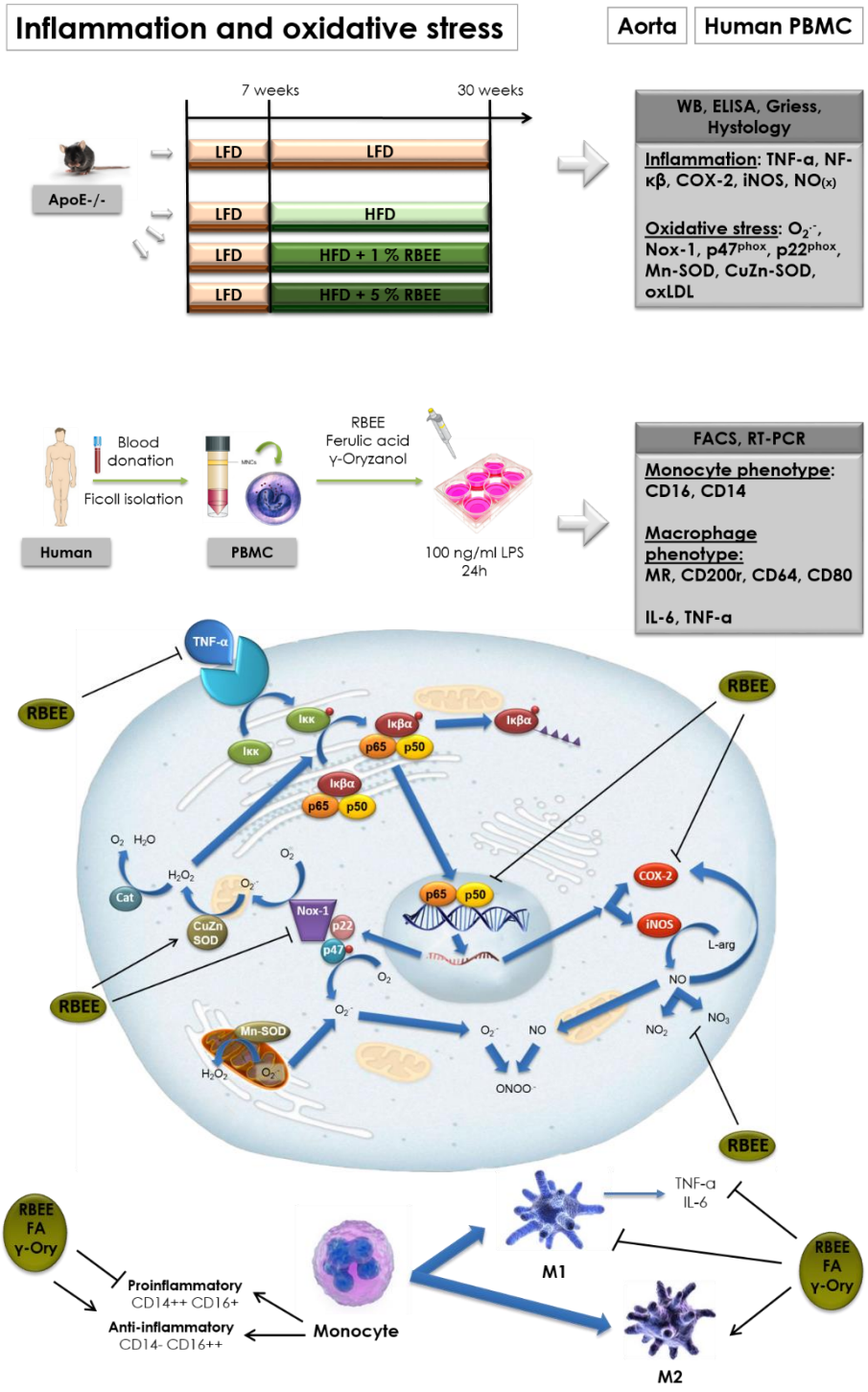


Figura 18: Diseño experimental y resumen de resultados.

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Atherosclerosis-related inflammation and oxidative stress are improved by rice bran enzymatic extract

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ABSTRACT

Rice bran has long been considered as a nutritional supplement in the prevention of cardiovascular diseases. ApoE−/− mice were fed high fat diet supplemented with 1 or 5% rice bran enzymatic extract (RBEE). In parallel, 100 ng/ml LPS induced human monocytes were treated with 20 μg/ml RBEE, ferulic acid (FA) or γ-oryzanol (γ-Ory). RBEE diet supplements reduced NF-κβ activation, TNF-α, COX-2 and iNOS expression and serum NO-derived metabolites. Additionally, NADPH oxidase subunits were downregulated, resulting in lower superoxide production, as evidenced by lower dihydroethidium fluorescence and oxLDL deposition in the aorta. RBEE, FA and γ-Ory reduced pro-inflammatory monocyte (CD14++ CD16+) phenotype and increased non-classical monocytes (CD14− CD16++). A shift towards M2 polarised macrophages was observed, leading to reduced IL-6 and TNF-α mRNA expressions. RBEE chronic consumption ameliorates atherosclerosis-related oxidative stress and inflammation showing its potential as nutritional supplement. FA moiety of γ-Ory was identified as the main responsible for the actions observed.

1. Introduction

Cardiovascular diseases, primarily atherosclerosis, are currently the leading cause of death worldwide. A vast amount of data supports the association between hypercholesterolaemia and atherosclerosis development (Rafieian-Kopaei, Setorki, Doudi, Baradaran, & Nasri, 2014). In addition to hypercholesterolaemia, inflammatory mechanisms coupled with oxidative stress play a key role in the atheroma formation process (Li, Horke, & Förstermann, 2014; Libby, 2012).

Moderate amounts of radical oxygen species (ROS) act as signalling molecules in several processes which include regulation of the vascular tone, the inflammatory response and apoptosis. However, imbalanced production of ROS originated by mitochondria, peroxisomes, lipoxygenases or NADPH

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E-mail address: cpternero@us.es (C. Perez-Ternero).
Chemical compounds: Ferulic acid (PubChem CID: 445858); γ-Oryzanol (PubChem CID: 51346127); β-Sitosterol (PubChem CID: 222284); γ-Tocotrienol (PubChem CID: 53394606).
http://dx.doi.org/10.1016/j.jff.2016.08.037
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oxidase (NADPHox), or deficient antioxidant scavenging from catalase, superoxide dismutase (SOD) or glutathione peroxidase, among others, results in oxidative stress causing dysfunctional signalling, damaged endothelium, and induction of pro-inflammatory responses. These events result in atherosclerosis promotion, among other cardiovascular diseases (Taverne, Bogers, Duncker, & Merkus, 2013).

Increased oxidative stress and high amounts of serum cholesterol trigger the pro-inflammatory response through cytokine release and nuclear factor κβ (NF-κβ) activation leading to leukocyte recruitment. Blood monocytes can extravasate into the tissue where they differentiate into classical (M1) or alternative (M2) activated macrophages. The relative relation between M1 and M2 subsets influences the progression of the plaque development (Woollard & Geissmann, 2010). M1 promotes the pro-inflammatory stimulus through cytokine release (e.g. IL-1β, IL-6, TNF-α, CCL2), ROS production and cholesterol uptake resulting in foam cell formation and unstable plaque growth. However, pro-resolving M2 polarised cells secrete anti-inflammatory IL-10 cytokine, contribute to tissue repair and promote the stabilisation of the plaque (Stöger et al., 2012).

Regarding the nutritional strategies to reduce high blood cholesterol, not only cholesterol-controlled diet but also bioactive components which help lower cholesterol are drawing the attention nowadays (Prasad, 2010). A number of recent studies point out the interest of nutritional agents such as polyphenols, tocols, phytosterols and γ-oryzanol (γ-Ory), naturally present in fruits, vegetables and grains, to considerably prevent and improve atherosclerosis disease due to lipid lowering, anti-inflammatory and antioxidant effects (Friedman, 2013; Goufo & Trindade, 2014; Islam et al., 2011).

Rice bran is a by-product of white rice milling which consists of the outer layer of the rice kernel (i.e. aleurone, pericarp and germ). This fraction is rich in interesting bioactive components such as γ-Ory, phytosterols and tocols. However, rice bran is currently underutilised due to rancidity led by endogenous lipases, which promote the hydrolysis of lipid components. This decomposition confers the characteristic bitter taste and rancid smell which impedes its consumption. Therefore, stabilisation aiming to prevent rancidity is of great interest to allow rice bran use by the food industry. The rice bran enzymatic extract (RBEE) used in this work was produced by an enzymatic method previously described (Parrado et al., 2006) which inactivates lipases and enables fully water solubilisation of its components due to the interaction of peptides with the lipophilic components. The enzymatic process also increases the amount and bioavailability of bioactive components compared to the raw material (Parrado et al., 2006). Owing to its composition rich in bioactive components, the RBEE used in this work has already shown its ability to reduce oxidative stress in a rat model of metabolic disease and to reduce plaque burden in ApoE–/– mice (Justo et al., 2013; Perez-Ternero, Herrera, Laufs, Alvarez de Sotomayor, & Werner, 2015).

Herein, we aimed to evaluate the effects of RBEE supplementation of high fat diet on the pro-inflammatory and prooxidant environment present in the vasculature of ApoE–/– mice. Additionally, we aimed to investigate if the activity of RBEE was comparable or higher than that already known of its main bioactive compounds in human PBMC.

2. Materials and methods

2.1. Animals and treatment

Male ApoE–/– mice on a C57BL/6J background were purchased from Charles River (Charles River Laboratories, L'Abresle, France). At seven weeks of age, mice were randomly separated into four groups (n = 10). One group was fed low fat diet (LFD, 2014, Harlan Laboratories, Madison, WI, USA) and the last three groups were fed high fat diet containing 0.2% (% kcal) cholesterol and 42% (% kcal) fat (HFD, TD 88137, Harlan Laboratories, Madison, WI, USA) supplemented or not with either 1% RBEE (HFD 1%) or 5% RBEE (HFD 5%) for 23 weeks. RBEE (1 and 5%; w/w) doses were chosen based on previous studies of our group where RBEE exerted atheroprotection in the ApoE–/– model and antioxidant and anti-inflammatory properties in the Zucker rat model of metabolic syndrome mice (Candiracci, Justo, Castaño, Rodriguez-Rodriguez, & Herrera, 2014; Justo et al., 2013; Perez-Ternero et al., 2015). All experimental procedures were approved by the University of Seville (Seville, Spain) Committee for Ethical Experimentation.

2.2. RBEE

Aiming at stabilisation and extraction of bioactive components, RBEE was prepared by endoprotease (trypsin- and chymotrypsin-like) enzymatic hydrolysis in a bioreactor pH (pH 8) and temperature (60 °C) controlled as previously described (Parrado et al., 2006), giving rise to a syrup completely soluble in water and rancidity free due to endogenous lipase inactivation. Bioactive components of raw rice bran and RBEE are available in Supplementary Table S1 and were analysed by HPLC and gaseous chromatography as previously described (Revilla et al., 2009). As a result of this extraction method, RBEE presents a rich nutraceutical composition: γ-Ory (8950 ± 850 mg/kg), phytosterols (3553 ± 66 mg/kg), ferulic acid (FA, 351 ± 5 mg/kg), tocotrienols (170 ± 15 mg/kg) and tocopherols (93.4 ± 10 mg/kg). Full description of RBEE and RB phytochemicals is available in Supplementary Table S1. The RBEE γ-oryzanol composition profile after enzymatic treatment was qualitatively similar to that of raw rice bran (cycloartenyl, 24-methylene cycloartenyl, campesteryl, and sitosteryl ferulates) but with two main differences: (i) the fat components are in a soluble form, which increases its bioavailability, and (ii) there is a specific enrichment in γ-oryzanol content.

2.3. Protein expression

Thoracic aortas were homogenised in lysis buffer containing: 50 mM Tris (pH 7.5), 8 mM $MgCl_2$, 5 mM EGTA, 0.5 mM EDTA, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, 0.01 mg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride, 250 mM NaCl and 1% Triton X-100 and phosphatase inhibitors. 15–50 μg of protein was resolved in 10% SDS–PAGE gels, transferred to polyvinylidene difluoride membranes and incubated with one of the following antibodies: anti-TNF-α (Santa Cruz Biotechnology, Heidelberg, Germany), anti-COX-2 (Cayman Chemical, Ann Arbor, MI, USA), anti-iNOS (Stressgen-Enzo Life Sciences, Farmingdale, NY, USA), anti-$p22^{phox}$ (Santa Cruz

Biotechnology, Heidelberg, Germany), anti-p47^{phox} (Santa Cruz Biotechnology, Heidelberg, Germany), anti-Nox-1 (Novus Biologicals, Cambridge, UK), anti-CuZn-SOD (Stressgen-Enzo Life Sciences, Farmingdale, NY, USA), anti-Mn-SOD (Stressgen-Enzo Life Sciences, Farmingdale, NY, USA) or anti-β-actin (Sigma, St. Louis, MO, USA). Antibody binding was visualised by enhanced chemiluminescence (Fujifilm LAS-3000 Imager) and quantification was performed with ImageJ software v1.47 (National Institutes of Health, Bethesda, MD, USA). Protein loading was normalised to β-actin and expressed as percentages of LFD values of the same gel.

2.4. *Nuclear NF-κβ activation*

p65 subunit of the NF-κβ complex was measured in 10 μg of protein of nuclear extracts from whole thoracic aorta homogenates using the ELISA-based TransAM NFκβ family kit (Active Motif, Rixensart, Belgium) as recommended by the manufacturer.

2.5. *$NO_{(x)}$ concentration measurement*

As NO itself is unstable *in vivo*, NO metabolites ($NO_{(x)}$) were measured using Griess reagent, as previously described (Granger, Taintor, Boockvar, & Hibbs, 1996). The measurements were made in serum from blood collected by intracardiac punction of anaesthetised mice prior to sacrifice and centrifuged (20 min, 1.500 *g*, RT).

2.6. *iNOS immunofluorescence*

Aortic arch was fixed with PFA and embedded in Tissue-Tek optimal cutting temperature compound. 10 μm thick sections were permeabilised with 0.25% Triton X-100/PBS, then blocked with 1% BSA for 30 min and incubated with anti-iNOS primary antibody (1:100, Pierce, Rockford, IL, USA) for 1 h at room temperature. After rinsing with PBS, tissue was incubated with Cy3 fluorescence secondary antibody (1:100, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Tissues without primary antibody were used as negative controls. A total of six sections per animal were acquired using an Olympus BX61 epifluorescence microscope (20× objective) and immunofluorescence images were analysed with ImageJ software v1.47 (National Institutes of Health, Bethesda, MD, USA) and normalised with the tissue area observed.

2.7. In situ *evaluation of vascular superoxide production*

The fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* production of superoxide (O_2^-) from frozen aortic arch sections. DHE is oxidised by superoxide to ethidium bromide, migrates to the nucleus where it intercalates within the DNA and emits an intense red fluorescence. Briefly, 14 μm thick serial sections from the aortic arch were equilibrated in Krebs–HEPES buffer. Then, slices were incubated with DHE (2 μmol/l) light protected in a humidified chamber, mounted and then visualised by confocal microscopy scanning (20× objective; Zeiss LSM 7 Duo; Zeiss, Oberkochen, Germany) using the same image settings for all the sections. Quantitative analysis of O_2^- production was performed with ImageJ software v1.47 (National Institutes of Health, Bethesda, MD, USA) and normalised with the tissue area observed.

2.8. *oxLDL measurement*

Aortic oxLDL was measured with a commercial ELISA kit (#Ref. SEA527Mu, Uscn Life Science Inc., Houston, TX, USA). Thoracic and abdominal aortas were excised and homogenised in lysis buffer of the same composition as listed before. Then, the assay was conducted as recommended by the manufacturer.

2.9. *Human blood collection and monocyte isolation*

Peripheral venous blood was isolated from five healthy adult volunteers (<35 years old) from the Hospital Virgen del Rocio at Seville. The investigation conformed to the principles outlined in the Helsinki Declaration of the World Medical Association and was conducted according to the guidelines of good clinical practice. Donors declared that they were non-smokers and were not taking any medication. Blood samples were collected into K_3EDTA-containing Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll Histopaque gradient (Sigma-Aldrich). Monocytes were then isolated from PBMC using positive selection with CD14 MicroBeads according to the manufacturer's instructions (MACS, Myltenyi Biotec, Madrid, Spain). Monocytes were tested for purity by CD14 fluorescein isothiocyanate labelling and fluorescence-activated cell sorter (FACS) analysis using a FACSCanto II flow cytometer and FACSDiva software (Becton Dickinson Immunocytometry Systems, CA, USA) (Varela et al., 2011). Following isolation, the cells were suspended in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, and 1% heat-inactivated FCS. The monocytes were used within 24 h after isolation for the experiments.

2.10. *Immunostaining analysis by FACS*

Membrane surface expression of CD16 (PE anti-human CD16; Miltenyi Biotec, Bergisch Gladbach, Germany) and CD14 (APC-Cy7 anti-human CD14, Miltenyi Biotec) in monocytes was assessed by FACS. A pilot study was performed in the range between 5 and 100 μg RBEE, FA or γ-Ory/ml and the dose of 20 μg/ml was selected. 5×10^5 of purified monocytes were stimulated *in vitro* with LPS for 24 h in the presence of either 20 μg/ml RBEE, FA or γ-Ory and then incubated with the above antibodies in the dark at room temperature for 15 min. Thereafter, cells were fixed and erythrocytes were lysed with a volume (20×) of FACS lysing solution (BD). Fluorescence intensity was measured in a FACSCanto II flow cytometer with a CellQuest software (BD). Results were analysed using the Win-List software package (Verity Software House; Topsham, ME, USA). Mean fluorescence intensity (MFI) of 10^4 counted cells was measured in each sample. Monocytes were gated as forward scatter^{high} (FSC^{high})-side scatter^{high} (SSC^{high}) cells. Expression levels were presented as MFI corrected for nonspecific binding of isotype control antibodies.

2.11. *RNA isolation and qRT-PCR analysis*

Total RNA was extracted from cells by using Trisure Reagent (Bioline, London, UK), as instructed by the manufacturer. RNA

quality was assessed by A260/A280 ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA (1 μg) was subjected to reverse transcription (iScript; Bio-Rad, Hercules, CA, USA) according to the manufacturers' protocol. An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a MX3000P system (Stratagene, Santa Clara, CA, USA). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad, Hercules, CA, USA) containing the primer pairs for the target gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin as housekeeping genes (Supplementary Table S2). All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers were used to calculate the relative mRNA expression using the standard 2–(ΔΔCt) method. All data were normalised to endogenous reference (GAPDH and β-actin) gene content and expressed as percentage of controls.

2.12. Statistics

Data were presented as mean ± SEM and n reflects the number of assays conducted. One-way ANOVA analysis was performed, followed by Bonferroni's test for multiple comparisons and statistical comparisons were evaluated using a GraphPad Prism Software v5.01 (San Diego, CA, USA). Pairwise comparisons were performed among all independent groups and results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. RBEE diet supplement reduced vascular inflammation

Transmembrane TNF-α is the precursor of the soluble active form of the pro-inflammatory cytokine TNF-α. Although transmembrane depots were not modified by the introduction of HFD (Fig. 1A), the soluble active form was upregulated (Fig. 1B, $p < 0.01$). 5% RBEE supplementation prevented this increase (Fig. 1B, $p < 0.05$). TNF-α is one of the most potent physiological inducers of NF-κβ by phosphorylation-dependent ubiquitination and degradation of Iκβ. Even though HFD did not induce further NF-κβ (p65) nuclear translocation, both doses of RBEE diet supplementation prevented NF-κβ activation (Fig. 1C, $p < 0.01$ *vs* HFD and $p < 0.05$ *vs* LFD).

Inflammation-related enzymes COX-2 and iNOS were upregulated by the introduction of the HFD (Fig. 1D and E, $p < 0{,}001$). Although 1% RBEE supplementation showed a reduction trend, only 5% RBEE supplements reduced its expression significantly ($p < 0.05$). As a consequence of the effects on iNOS expression, the introduction of HFD greatly increased $NO_{(x)}$ concentration (Fig. 1F, $p < 0.001$). Both 1 and 5% RBEE supplements reduced NO-derived species ($p < 0.05$). To better confirm iNOS expression in the aortic tissue, especially in the aortic arch which is one of the most susceptible areas to atherosclerosis development, immunofluorescence staining for iNOS was performed in this tissue. Here, iNOS expression was found to be enhanced by the HFD (Fig. 2A and C, $p < 0.001$) and prevented by the RBEE diet supplementation regardless of the dose (Fig. 2D and E, $p < 0.01$ and $p < 0.05$ *vs* HFD for 1 and 5%, respectively).

3.2. Diet supplementation with 1% RBEE reduced vascular oxidative stress

HFD increased superoxide production in the aortic arch ($p < 0.05$), which was counteracted by 1% RBEE diet supplementation (Fig. 3A–D, $p < 0.05$). 5% RBEE supplements to HFD showed a protective trend but did not reach statistical significance (Fig. 3E).

To investigate the mechanism by which RBEE reduced superoxide anion production, NADPHox subunits (main source of superoxide anion) and SOD isoforms (major superoxide scavenger in the cell) protein expressions were evaluated in the aorta. HFD induced the expression of all NADPHox subunits (Fig. 4A–C, $p < 0.001$ for Nox-1 and $p < 0.01$ for $p47^{phox}$ and $p22^{phox}$). RBEE diet supplementation reduced Nox-1 (Fig. 4A, $p < 0.05$) and $p47^{phox}$ (Fig. 4B, $p < 0.01$) expressions regardless of the dose. However, only 1% diet supplementation was able to reduce $p22^{phox}$ expression (Fig. 4C, $p < 0.01$). With regard to SOD isoforms, mitochondrial Mn-SOD was upregulated by HFD (Fig. 4D, $p < 0.01$), without any effect introduced by RBEE supplementation. Nevertheless, although HFD did not change CuZn-SOD expression level, 1% RBEE supplements upregulated its expression (Fig. 4E, $p < 0.05$).

3.3. RBEE prevented oxLDL accumulation in the aorta

OxLDL was measured in aortic homogenates in order to assess the uptake by macrophages (Fig. 4F). HFD fed animals showed increased accumulation of oxLDL in the aorta ($p < 0.05$), which was dose-dependently reduced by RBEE (38.8% and 81.7% reduction for HFD 1% [$p < 0.05$ *vs* HFD] and HFD 5% [$p < 0.001$ *vs* HFD], respectively).

3.4. RBEE modulates monocyte phenotype and macrophage polarisation in human PBMC

The absolute count for peripheral blood monocytes was not different among the 5 groups (data not shown). The distribution of CD14 and CD16 as different human monocyte subsets was determined by flow cytometry. We observed that CD14++ CD16– monocyte subsets exposed to LPS (100 ng/ml) were not significantly different from the rest of the groups (Fig. 5A); however, LPS significantly increased the expression of CD14++ CD16+ intermediate monocyte subsets compared to control (Fig. 5B, $p < 0.001$). Treatment with RBEE, FA or γ-Ory did not only abrogate the effect caused by LPS on intermediate monocytes but also increases the levels of non-classical monocytes (CD14– CD16++), where RBEE achieved the highest bias to the aforementioned non-classical monocyte subset (Fig. 5C, $p < 0.001$). Latter, we analysed the ability of RBEE, FA and γ-Ory to sensitise monocytes for polarisation towards M1 or M2 macrophages. To approach this, gene expression of phenotypic markers for M1 (CD64, CD80) and M2 (MR, CD200r) subsets were examined by real time PCR in monocytes exposed for 24 hours to LPS in the presence or absence of RBEE, FA or γ-Ory. MR and

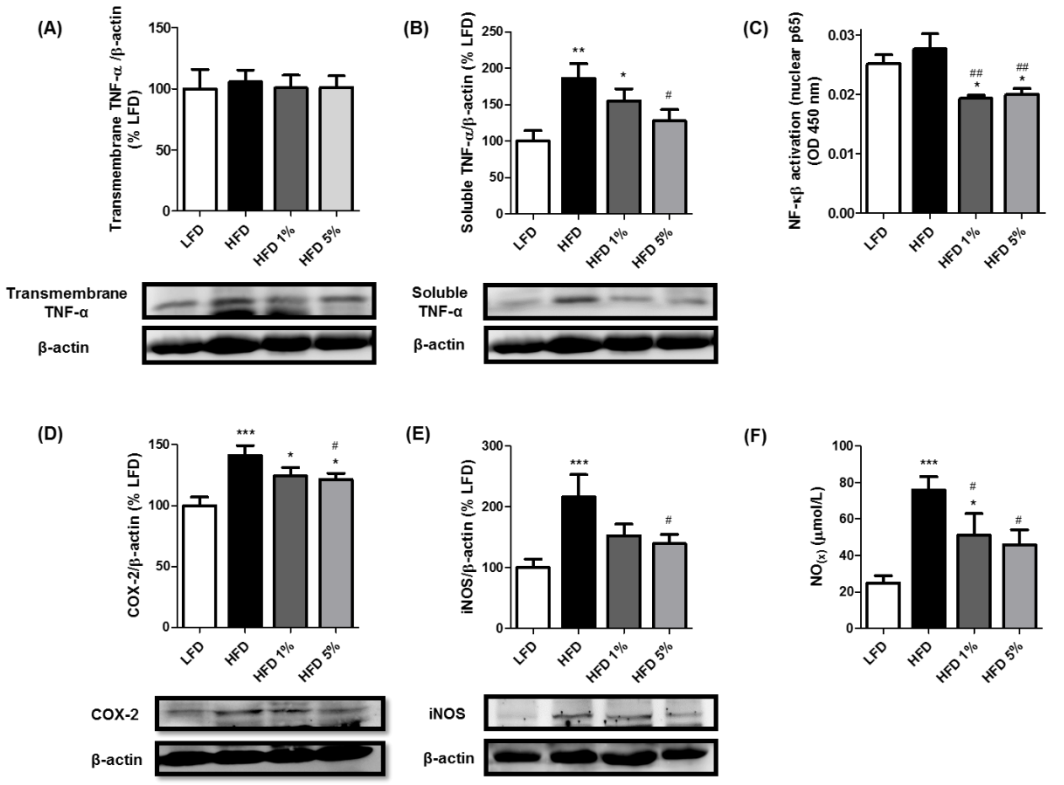

Fig. 1 – RBEE diet supplementation reduces inflammation. Western blot analysis of transmembrane TNF-α (A), soluble TNF-α (B), NF-κβ (p65) nuclear quantification (C), COX-2 (D) and iNOS (E) in the aortas and serum concentration of NO-derived species (F) of animals fed low (LFD), high fat (HFD) and 1 and 5% RBEE supplemented diets (HFD 1% and HFD 5%). Data represent the mean ± SEM of the data from 6–9 animals in each group. *p < 0.05, **p < 0.01 and ***p < 0.001 *vs* LFD; #p < 0.05 and ##p < 0.01 *vs* HFD.

CD200r were downregulated by LPS in circulating monocytes (Fig. 5D and E, $p < 0.001$ and $p < 0.05$, respectively); however, the presence of RBEE, FA and γ-Ory abrogated the effect of this latter by increasing the mRNA levels of M2 markers, which were slightly higher than those found in the untreated control. Unlike what happened to MR and CD200r, M1 markers CD64 and CD80 were upregulated after LPS incubation (Fig. 5F and G, $p < 0.001$). As mentioned above, RBEE, FA and γ-Ory reversed the phenotype of LPS-treated monocytes diminishing the mRNA levels of CD64, and CD80 ($p < 0.001$), with RBEE being significantly lower than the rest of the tested compounds. Since M1 macrophages are characterised by their ability to secrete pro-inflammatory cytokines, we next tested whether our treatments were able to modulate the gene expression of pro-inflammatory cytokines such as IL-6 or TNF-α. As expected and in line with the data above, RBEE, FA and γ-Ory resulted in a massive reduction of IL-6 (Fig. 5H, $p < 0.001$) and TNF-α (Fig. 5I, $p < 0.001$) compared to LPS-treated monocytes, yielding values close to untreated control.

4. Discussion

Hyperlipidaemia and inflammation trigged by oxidative stress are on the root of the pathological process of atherosclerosis. A number of clinical and animal studies have shown the potential therapeutic value of rice bran by-products in atherosclerosis and other cardiovascular diseases (Cicero & Gaddi, 2001; Friedman, 2013; Goufo & Trindade, 2014; Islam et al., 2011). However, the exact components responsible for single actions remain unclear. In this study, we showed for the first time in the ApoE−/− mice model of atherosclerosis that RBEE diet supplementation modulates inflammation by reducing TNF-α activation of NF-κβ and downstream pro-inflammatory enzymes COX-2 and iNOS. The antioxidant potential of RBEE revealed by lower superoxide production was confirmed by reduced expression of NADPHox subunits (Nox-1, $p47^{phox}$ and $p22^{phox}$) and increased expression of CuZn-SOD, resulting in lower oxLDL uptake in the aorta (Fig. 6). RBEE

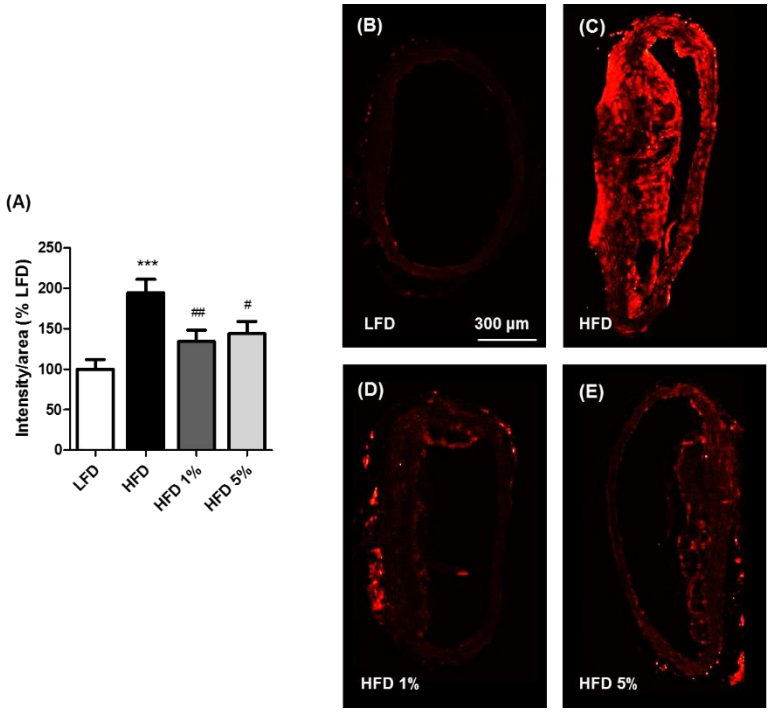

Fig. 2 – RBEE diet supplementation downregulates iNOS expression. iNOS immunofluorescence analysis (A) of the aortas of animals fed low (LFD, B), high fat (HFD, C) and 1 and 5% RBEE supplemented diets (HFD 1% [D] and HFD 5% [E]). Scale bar indicates 300 µm. Data represent the mean ± SEM of the data from 6–7 animals in each group. $^{***}p < 0.001$ *vs* LFD; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs* HFD.

was also able to modulate monocyte phenotype and to induce a shift towards M2 subset, being the effects higher than those observed for FA and γ-Ory.

The classical activation of the canonical NF-κβ pathway can be initiated by a number of extracellular stimuli, including cytokines such as TNF-α through phosphorylation of Iκκ and Iκβα resulting in translocation of p65-p50 complex to the nucleus. Once activated, p65-p50 can selectively induce the transcription of pro-inflammatory enzymes including COX-2 and iNOS (Lawrence, 2009). While COX-2 mediates pro-inflammatory signalling by prostaglandins and thromboxane production, iNOS is responsible for overproduction of NO which can further react with superoxide resulting in highly reactive oxygen radicals, which may damage DNA, lipids and proteins (Buttery & Polak, 1999). Here, the introduction of HFD induced the inflammatory response evidenced by higher expression of soluble TNF-α, COX-2 and iNOS, which led to increased production of NO species. Supplementation of the diet with RBEE inhibited the whole cascade of NF-κβ activation, reducing in turn the expression of COX-2 and iNOS enzymes and serum NO species. Accordingly, we have previously proved the anti-inflammatory potential of RBEE diet supplementation in the vasculature and adipose tissue of Zucker rats which develop metabolic syndrome (Candiracci et al., 2014; Justo et al., 2013, 2015) and in high fat diet-induced obese mice by a mechanism related to TNF-α release and iNOS and COX-2 expressions. On the same line, others demonstrated that FA and γ-Ory from RBEE have anti-inflammatory potential by reduction of NF-κβ activation and pro-inflammatory cytokine release (Akihisa et al., 2000; Islam et al., 2011; Sakai et al., 2012). It is noteworthy to mention that the hypolipidaemic activity of several components of rice bran, such as phytosterols and γ-Ory, also have a positive effect on inflammation as reviewed elsewhere (Islam et al., 2011; Tall & Yvan-Charvet, 2015).

An imbalance between ROS production and scavenging causes ROS accumulation leading to oxidative stress and inflammation, which are involved in every step of atherosclerosis development, including endothelial damage, macrophage chemotaxis, foam cell formation and smooth muscle cell migration and proliferation (Stocker & Keaney, 2004). Along with the mitochondria, NADPH oxidase is the main source of superoxide in the cell. HFD induced oxidative stress as shown by increased expression of all NADPHox subunits resulting in increased superoxide release evidenced by DHE staining. Mn-SOD acts as a local scavenger in the mitochondria, which produces huge amounts of ROS as a by-product of oxidative phosphorylation. In accordance with our findings, compensatory overexpression of Mn-SOD, aiming to reduce local oxidative

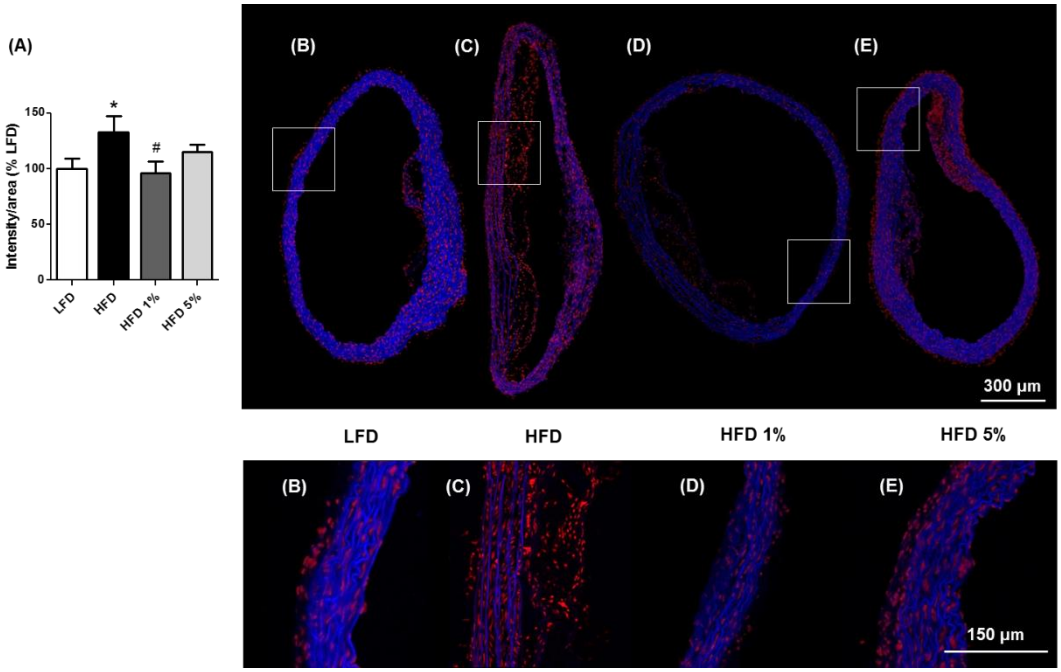

Fig. 3 – Superoxide anion release is reduced by RBEE. Quantification (A) and representative pictures of dihydroethidium staining of the aortas of animals fed low (LFD, B), high fat (HFD, C) and 1 and 5% RBEE supplemented diets (HFD 1% [D] and HFD 5% [E]). Scale bars indicate 300 µm and 150 µm. Data represent the mean ± SEM of the data from 5–6 animals in each group. $^{*}p < 0.05$ *vs* LFD and $^{\#}p < 0.05$ *vs* HFD.

stress, was found in mice under high fat diet (Ruggiero, Ehrenshaft, Cleland, & Stadler, 2011). With regard to NADPHox, RBEE diet supplementation reduced the expression of Nox-1 and $p47^{phox}$ subunits regardless of the dose. Additionally, 1% RBEE supplements reduced $p22^{phox}$ subunit and increased cytoplasmic CuZn-SOD, explaining further antioxidant protection as evidenced by reduced superoxide production measured by DHE staining. We and others have previously demonstrated the potential of RBEE, rice bran by-products and isolated rice bran components to reduce oxidative stress *in vivo* in atherosclerosis and different cardiovascular models, revealing tocotrienols, FA and γ-Ory as the most potent antioxidant molecules present in rice bran (Jin Son, Rico, Hyun Nam, & Young Kang, 2010; Justo et al., 2013; Kwon et al., 2010; Masisi, Beta, & Moghadasian, 2016; Qureshi, Salser, Parmar, & Emeson, 2001; Qureshi, Sami, Salser, & Khan, 2002). It is noteworthy that 1% RBEE diet supplementation exerted higher antioxidant protection than 5%. This effect could be explained since the higher dose of RBEE supplies a higher amount of tocotrienols. Although both tocotrienols and tocopherols present in RBEE exert antioxidant properties, tocotrienols can be converted to tocopherols *in vivo*, which at high dose may become prooxidant (Bowry & Stocker, 1993; Khor, Chieng, & Ong, 1995). This dual effect may explain that at a higher dose, as that provided by HF 5% diet, the antioxidant protection is lost (Temelkan, Bilge Yıldoğan, İnci, Filiz, & Reşat, 2013).

In healthy subjects, there is a balanced flow of LDL particles between the arterial wall and blood stream. This diffusion increases proportionally with hypercholesterolaemia. Moreover, under endothelium-damaged conditions, the arterial wall increases its permeability, LDL uptake is augmented and the particles are oxidised activating the NF-κβ system. The inflammatory response induced results in monocyte recruitment and macrophage differentiation, which after cholesterol phagocytosis become into foam cell, the fatty core of the atherosclerotic plaque (Pirillo, Norata, & Catapano, 2013). In this work, the increase of oxLDL found in the arterial wall after HFD consumption was dose dependently reduced by RBEE. Although oxidative stress was lower in 1% RBEE group, the serum cholesterol concentration was found to be lower in 5% RBEE mice in another study performed with these mice under the same conditions (Perez-Ternero et al., 2015). The higher consumption of phytosterols, which are known to disrupt cholesterol absorption in the gut, could explain this improvement in the serum profile. Since the LDL deposition in the arterial wall is gradient dependent, lower serum cholesterol would rebound in lower LDL in the arterial wall, where it is oxidised.

Macrophages orchestrate the inflammatory response in atherosclerosis and are vulnerable to changes in their microenvironment such as redox state or cytokines, modifying

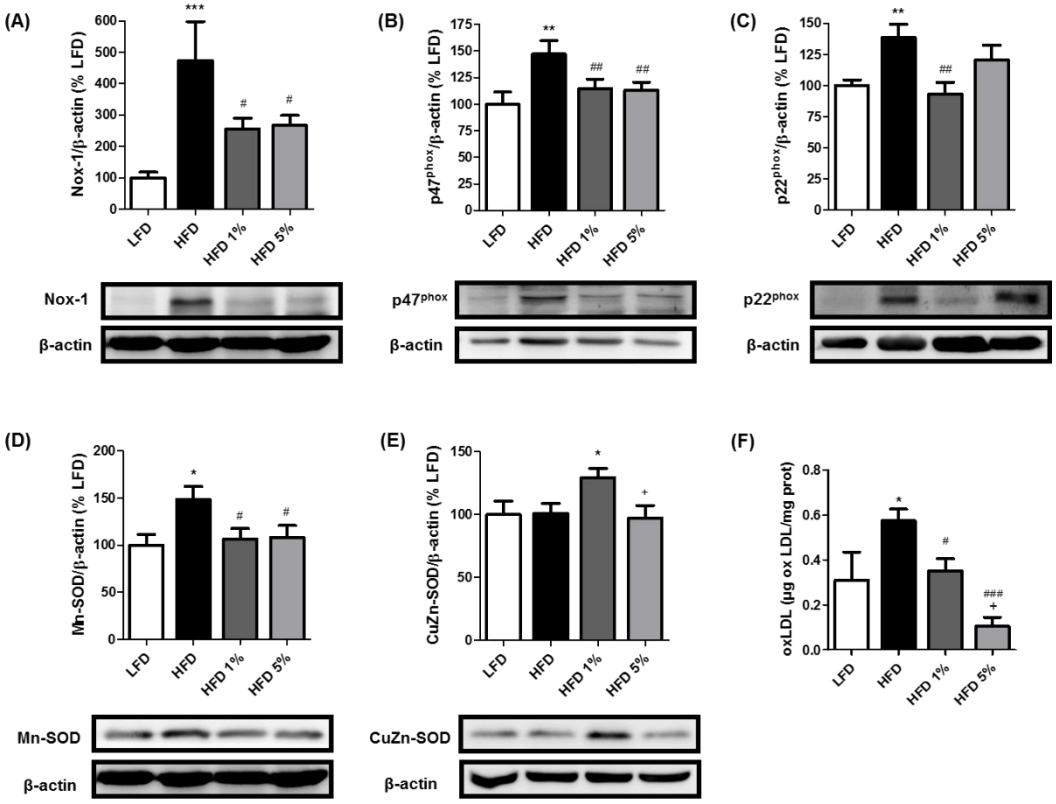

Fig. 4 – RBEE protects against oxidative stress. Western blot analysis and representative images of Nox-1 (A), p47^{phox} (B), p22^{phox} (C), Mn-SOD (D) and CuZn-SOD (E) and oxLDL uptake quantification in the aortas of animals fed low (LFD), high fat (HFD) and 1 and 5% RBEE supplemented diets (HFD 1% and HFD 5%). Data represent the mean ± SEM of the data from 6–9 animals in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* LFD; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* HFD and $^{+}p < 0.05$ *vs* HFD 1%.

their phenotypes and functions accordingly (Chinetti-Gbaguidi, Colin, & Staels, 2015). Consequently, the relative content in classical (M1) and alternative (M2) activated macrophages determines the progression and stability of the atherosclerotic plaque (Cho et al., 2013). RBEE modulated human PBMC to a lesser inflammatory phenotype and induced pro-resolving M2 polarisation shift at the concentration assayed, which came along with a sharply reduction of pro-inflammatory cytokines IL-6 and TNF-α. Even though FA and γ-Ory showed a remarkable effect, it was RBEE who had the greater impact on monocyte activation, even though the concentrations of γ-Ory and FA found in the RBEE are much lower than that assayed here, highlighting a possible synergistic effect or that the interaction between the components in the extract favours the absorption. Therefore, RBEE shows interest over the isolated components. In previous studies, FA was proved to modulate *in vitro* the inflammatory response by inhibition of LPS-induced macrophage inflammatory protein-2 (MIP-2) and TNF-α production (Sakai, Ochiai, Nakajima, & Terasawa, 1997). γ-Ory is a mixture of ferulic acid esters of sterols and triterpene alcohols. Therefore, our results might be mainly attributed to FA since it is part of the γ-Ory molecule. Many studies have aimed to describe γ-Ory kinetics of absorption and metabolisation, but the whole molecule has not been yet identified in plasma in contrast to FA alone (Fujiwara, Sakurai, Sugimoto, & Awata, 1983; Pan, Cai, & He, 2014). We hypothesise that γ-Ory is metabolised by esterases in the gut and FA is set free to be absorbed while triterpenic alcohols remain in the intestines acting as hypolipidaemic molecules.

Taken together, these results suggest that chronic consumption of RBEE can help ameliorate pro-inflammatory state present in atherosclerosis disease through downregulation of the NF-κβ signalling pathway and modulation of monocyte phenotype. The fact that RBEE is capable of reaching greater effects points out its interest as a nutritional supplement against atherosclerosis-related inflammation and oxidative stress.

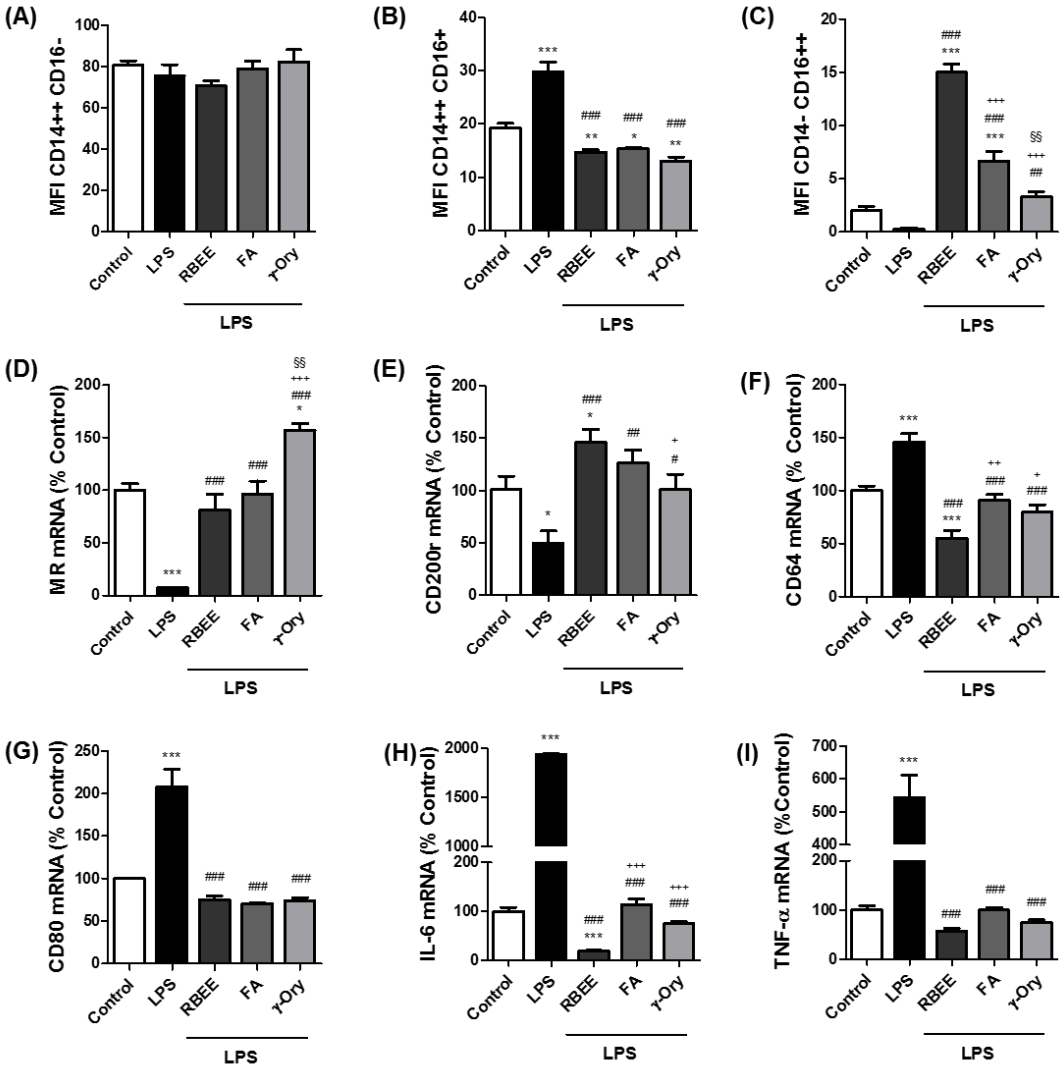

Fig. 5 – RBEE, FA and γ-Ory modulate macrophage response. Human monocytes were exposed to LPS (100 ng/ml) in the presence or absence of either 20 μg/ml of RBEE, FA or γ-Ory. Classical CD14++ CD16– (A), intermediate CD14++ CD16+ (B) and non-classical CD14– CD16++ (C) monocyte subsets were evaluated by flow cytometry. Gene expression of phenotypic markers for M2 (MR [D] and CD200r [E]) and M1 (CD64 [F] and CD80 [G]) macrophage subsets and pro-inflammatory cytokines (IL-6 [H] and TNF-α [I]) were examined by real time PCR. Data represent the mean ± SEM of the data from 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* LPS, $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.0001$ *vs* RBEE and $^{\S\S}p < 0.01$ *vs* FA.

Statements

- All of the authors listed have contributed to the work and have read, participated in the writing and agreed with the submitted version of the manuscript.
- The document is original and it has not been published or submitted elsewhere.
- The animal studies performed throughout the investigation have been reviewed by the appropriate Ethics Committees.

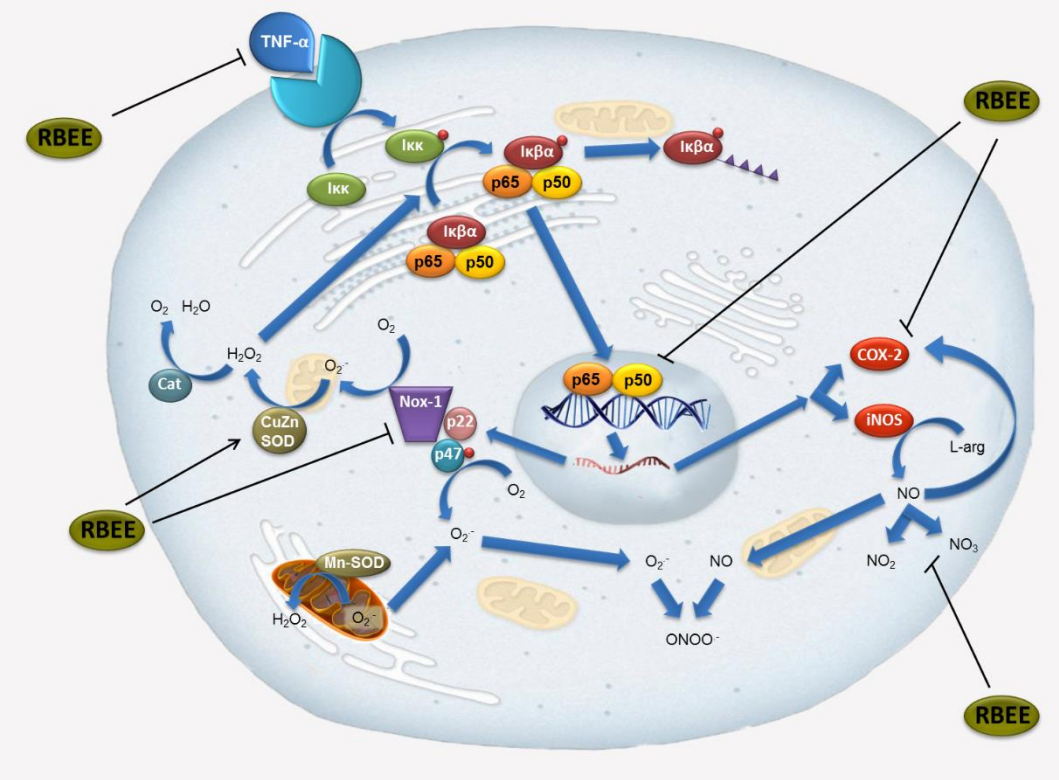

Fig. 6 – Mechanism of action of RBEE in the vasculature of ApoE–/– mice. RBEE reduced TNF-α expression, leading to lower NF-κβ activation and transcription of pro-inflammatory enzymes COX-2 and iNOS as well as NO release. RBEE prevented increased oxidative stress induced by HFD by upregulation of CuZn-SOD expression and downregulation of NADPH oxidase subunits.

- The investigation with human PBMC conformed to the principles outlined in the Helsinki Declaration of the World Medical Association and was conducted according to the guidelines of good clinical practice.

Conflict of interest

The authors have no relevant conflict of interest to disclose.

Acknowledgements

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.08.037.

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Supplementary table 1: Composition of rice bran before and after enzymatic extraction
Energy density, dietary macronutrients and bioactive composition of raw rice bran (RB) and rice bran enzymatic extract (RBEE).

Nutrient	RB	RBEE
Energy (kcal/g)	4.75	5.49
Protein (% kcal)	14.3	24.7
Carbohydrate (% kcal)	43,0	21.1
Fat (% kcal)	42.6	54.2
Saturated (% fat)	18.3	20.4
Monounsaturated (% fat)	43.1	42.4
Polyunsaturated (% fat)	37.2	35.8
Unknown	1.34	1.40
Phytosterols (mg/kg)	4140 ± 38	3553 ± 66
Brassicasterol	15 ± 1.1	15 ± 1.0
2,4 Metilencholesterol	8.7 ± 0.9	6.9 ± 3.0
Campesterol	1073 ± 22	871 ± 23
Campestarol	50 ± 3.3	41 ± 3.0
Stigmasterol	640 ± 22	504 ± 28
δ-7-campesterol	58 ± 5.4	18 ± 2.0
Clerosterol	50 ± 6.7	58 ± 5.0
β-Sitosterol	1784 ± 19	1694 ± 32
Sitostanol	63 ± 3.3	58 ± 4.0
δ-Avenasterol	103±5.6	127 ± 9.0
δ-5-2,4-Stigmasterol	23 ± 5.6	32 ± 2.3
δ-7-Stigmasterol	99 ± 8.7	17 ± 2.0
δ-7-Avenasterol	53 ± 4.9	18 ± 1.5
Others	91 ± 7.4	73 ± 6.2
γ-oryzanol (mg/kg)	7100 ± 0.40	8950 ± 0.85
Tocotrienols (mg/kg)	137 ± 15	170 ± 15
α-tocotrienol	45.4 ± 5.0	32 ± 2.0
γ-tocotrienol	88.6 ± 7.0	132 ± 9.0
δ-tocotrienol	3.8 ± 0.3	5.9 ± 0.3
Tocopherols (mg/kg)	125 ± 12	93.4 ± 10
α-tocopherol	76.7 ± 6.0	38.6 ± 4.0
β-tocopherol	7.2 ± 5.0	9.9 ± 0.5
γ-tocopherol	27.2 ± 3.0	20.4 ± 3.0
δ-tocopherol	14.6 ± 2.0	24.4 ± 4.0

Supplementary table 2: Primer sequence used for real-time PCR.

Target	GenBank accession number	Direction	Sequence (5'→3')
CD80	NM_005191.3	Forward Reverse	GGGGAAATGTCGCCTCTCTG GATTGGAGGGTGTTCCTGGG
CD64	NM_000566.3	Forward Reverse	TGTCATGCGTGGAAGGATAA TGTCACAGATGCATTCAGCA
MRC-1	NM_002438.3	Forward Reverse	GGCGGTGACCTCACAAGTAT ACGAAGCCATTTGGTAAACG
CD200r	NM_138939.2	Forward Reverse	GTTGCCCTCCTATCGCATTA TGGAAATTCCCATCAGGTGT
TNF-α	NM_000594.3	Forward Reverse	TCCTTCAGACACCCTCAACC AGGCCCCAGTTTGAATTCTT
IL-6	NM_000600.3	Forward Reverse	TACCCCCAGGAGAAGATTCC TTTTCTGCCAGTGCCTCTTT
GAPDH	NM_001289746.1	Forward Reverse	GAGTCAACGGATTTGGTCGT TTGATTTTGGAGGGATCTCG
b-actin	NM_001101.3	Forward Reverse	CGCAAAGACCTGTATGCCAA CACACAGAGTACTTGCGCTC

CAPÍTULO V

Uno suele encontrar su destino en el camino que toma para evitarlo

(Ooway, Kung Fu Panda, 2008)

LA SUPLEMENTACIÓN DIETÉTICA CON EL EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ PREVIENE LA APOPTOSIS VASCULAR Y LA ATEROSCLEROSIS EN RATONES APOE-/-

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Eur J Nutr. 2015. doi:10.1007/s00394-015-1074-z

La enfermedad aterosclerótica está asociada con una reducción en la longitud de los telómeros de las células mononucleares (MNC) y con un incremento de la senescencia celular en las placas ateroscleróticas. El salvado de arroz es una excelente fuente de ácido ferúlico, γ-oryzanol, fitosteroles y tocoles, los cuales tienen propiedades hipolipemiantes, antioxidantes y antiinflamatorias. El objetivo de este trabajo fue la evaluación del impacto de la suplementación dietética con un extracto enzimático de salvado de arroz (EESA) sobre la apoptosis, la longitud de los telómeros y el desarrollo del proceso aterosclerótico en ratones deficientes en apolipoproteína E (ApoE-/-).

Ratones ApoE-/- de 7 semanas de edad fueron alimentados durante 23 semanas con una dieta alta en grasa y colesterol (HFD), suplementada o no al 5% con EESA. Como grupo control se emplearon ratones de genotipo salvaje (C57BL/6J) alimentados con dieta baja en grasas y colesterol (LFD). Aorta, suero y MNC aisladas del bazo mediante separación por gradiente de densidad con Ficoll fueron congelados para su posterior análisis.

El tratamiento con EESA redujo los valores séricos de colesterol total y triglicéridos y aumentó los valores de colesterol HDL. Además, el EESA atenuó la infiltración de macrófagos un y el desarrollo de la placa aterosclerótica en la válvula aórtica. En la aorta, el tratamiento con EESA inhibió la expresión de moléculas relacionadas con la apoptosis y senescencia celular: p16, p53 y el ratio bax/bcl-2. Como consecuencia, se redujo la apoptosis de células endoteliales. Sin embargo, las MNC de los grupos tratados con ESSA mostraron una mayor expresión de los marcadores de apoptosis p53 y del ratio bax/bcl-2. En comparación con el grupo control C57BL/6J, los ratones ApoE-/- mostraron un acortamiento de los telómeros en la aorta y MNC, que fue contrarrestado mediante la suplementación de la dieta con EESA. La suplementación de la dieta con EESA también incrementó la expresión de la proteína protectora de los telómeros TRF2.

Con todo esto, podemos confirmar que la suplementación de la dieta con EESA reduce el colesterol y previene el desarrollo de la placa aterosclerótica en ratones ApoE-/- y que en dicho proceso se han identificado una regulación diferente de procesos de senescencia celular y apoptosis en aorta y MNC.

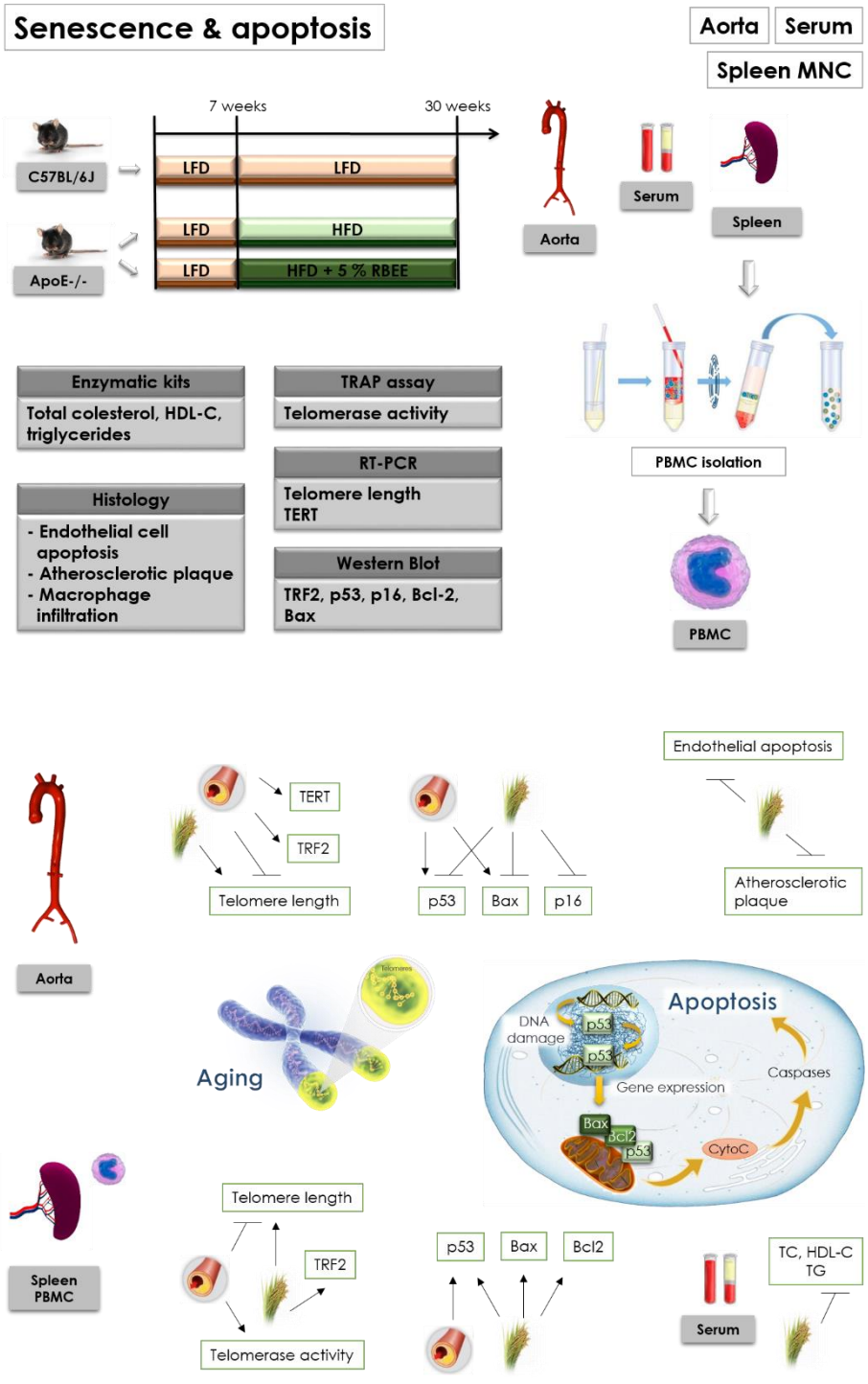

Figura 19: Diseño experimental y resumen de resultados.

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ORIGINAL CONTRIBUTION

Food supplementation with rice bran enzymatic extract prevents vascular apoptosis and atherogenesis in ApoE−/− mice

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Abstract
Purpose Atherosclerosis is associated with reduced mononuclear cell (MNC) telomere length, and senescent cells have been detected in atherosclerotic plaques. Rice bran is a source of γ-oryzanol, phytosterols and tocols with potential lipid-lowering, antioxidant and anti-inflammatory activities. Here, we tested the hypothesis that rice bran enzymatic extract (RBEE) impacts on apoptosis, telomere length and atherogenesis in mice.
Methods Seven-week-old male ApoE−/− mice were fed high-fat diet (HFD) or isocaloric HFD supplemented with 5 % (w/w) RBEE for 23 weeks. Wild-type mice of the same age were kept under standard diet as controls.
Results RBEE treatment reduced total cholesterol (19.24 ± 1.63 vs 24.49 ± 1.71 mmol/L) and triglycerides (1.13 ± 0.18 vs 1.75 ± 0.22 mmol/L) and augmented HDL-cholesterol (1.86 ± 0.20 vs 1.07 ± 0.20 mmol/L). RBEE attenuated macrophage infiltration by 56.69 ± 4.65 % and plaque development (7737 ± 836 vs 12,040 ± 1001 μm^2) in the aortic sinus. In the aorta, RBEE treatment reduced expression of the apoptosis pathway components p16, p53 and bax/bcl-2 ratio. RBEE prevented apoptosis of aortic endothelial cells (2.81 ± 0.71–1.14 ± 0.35 apoptotic nuclei/ring for ApoE−/− HFD and ApoE−/− HFD 5 % RBEE, respectively). In contrast, MNC of RBEE-fed mice exhibited enhanced apoptosis marker expression with increased p53 and bax/bcl-2 protein levels. Compared to WT, ApoE−/− mice on HFD were characterized by significant telomere shortening in aorta (11 ± 2 %) and MNC (73 ± 7 %), which was reduced by supplementation with RBEE (aorta: 40 ± 7 %; MNC: 105 ± 10 %). Expression of telomere repeat-binding factor 2 was increased in RBEE-fed mice.
Conclusion Long-term food supplementation with RBEE lowers cholesterol and prevents atherosclerotic plaque development in ApoE−/− mice. Differential regulation of vascular and MNC apoptosis and senescence were identified as potential mechanisms.

Keywords ApoE−/− · Rice bran enzymatic extract · Atherosclerosis · Inflammation · Apoptosis · Telomeres

Introduction

Atherosclerosis is a state of chronic low-grade inflammation and increased vascular oxidative stress. In addition, atherosclerosis is an age-related degenerative disease [1]. On the cellular level, aging is controlled by senescence regulators including telomeres and telomere-associated proteins [2]. Telomere dysfunction has been linked to the pathogenesis of cardiovascular disease [3], but to what extent telomere shortening is a consequence or causally associated with the onset of cardiovascular disease remains yet to be fully understood.

Endothelial injury, inflammation and pathologic vascular cell growth during atherosclerotic plaque generation are

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associated with higher rates of cellular turnover. Telomeres are regions of nucleotide repeats at the end of each chromosome which are shortened during every cell replication in a process that is enhanced by oxidative stress and inflammation, leading to senescence and potentially apoptosis [4]. Therefore, vascular telomere length may serve as an indicator of endothelial health. We have previously observed that telomere-regulating proteins are critical for endothelial stress resistance and protection, e.g., mediated by exercise or PPAR agonists [5, 6].

Shortened telomeres have been reported in aortic tissue of patients suffering from cardiovascular disease, indicating cellular senescence as a predictor of the onset, development and prognosis of atherosclerotic disease [7]. Moreover, telomere length in atherosclerotic plaques has been found to be altered depending on plaque characteristics [8]. It has been suggested that leukocyte telomere length is a biomarker of vascular aging, because in clinical studies atherosclerosis was found to be related to telomere shortening of leukocytes [9–12]. In fact, shorter leukocyte telomere length is associated with all major atherosclerosis risk factors such as age, diabetes, obesity, hypertension and smoking [13]. On the other hand, during atherogenesis, mononuclear cell (MNC) telomere exhaustion may serve as a mechanism of protection from atheroma progression by limiting inflammatory cell proliferative potential [14]. In this respect, lipid-modifying strategies may be important, as certain lipoproteins—especially LDL-cholesterol—are pro-inflammatory and trigger and sustain atherosclerosis. In addition to known lipid-lowering drugs (e.g., statins), lifestyle changes including diet may be important to attenuate or delay atherosclerosis [15–17].

Rice bran (RB) is a by-product of rice milling consisting of the aleurone layer and some parts of the endosperm and germ, rich in γ-oryzanol, tocols (including tocopherols and tocotrienols) and phytosterols, among others [18]. In spite of its medical potential due to a high content in antioxidant, lipid-lowering and anti-inflammatory components [19–21], it is currently primarily utilized for animal feeding. The insolubility and high rancidity of previous RB preparations are due to a high content in fatty components and hamper its use by the human food industry. The novel rice bran enzymatic extract (RBEE) used in this work avoids such drawbacks since it is a syrup with an enriched profile of nutraceutical components (i.e., 3.4-fold γ-oryzanol and threefold tocols) completely soluble in water and non-rancid due to the use of an endoprotease extraction method [22].

Here, we examined in the ApoE−/− mouse model of high-fat diet (HFD)-induced atherogenesis whether long-term RBEE supplementation confers anti-atherosclerotic effects and RBEE effect on apoptosis, telomeres and markers of cellular aging in the aorta and in MNC of HFD-fed ApoE−/− mice.

Materials and methods

Animals and diets

Six-week-old male apolipoprotein E knockout (ApoE−/−) mice on a C57BL/6J background and wild-type (C57BL/6J) mice were purchased from Charles River Laboratories (L'Abresle, France). The number of animals is indicated in the respective figure legends and ranges from 5 to 15 depending on the assay. The animals were housed under a 12/12-h light/dark cycle in a temperature-controlled environment (25 °C) and provided food and tap water ad libitum. At 7 weeks of age, ApoE−/− mice were switched from normal chow diet to high-fat diet (Harlan Laboratories, Madison, WI, USA) containing 0.2 % (% kcal) cholesterol and 42 % (% kcal) fat and randomly separated into two groups: HFD and HFD 5 % RBEE, whose diet was supplemented with 5 % (w/w) of RBEE. Final nutrient composition in fat, protein and carbohydrates did not vary with this supplementation (Supplementary Table 1). At 30 weeks of age, mice were anesthetized with high-dose pentobarbital and killed by exsanguination. As non-atherosclerotic control, C57BL6/J mice were kept on normal chow diet for the same period. After killing and spleen homogenization, splenic MNC was isolated by Ficoll density-gradient centrifugation using standard protocols. Approval for all experiments was provided by the Ethic Committee for Animal Experimentation of the University of Seville (Spain) (AGL2013-407791-P).

Table 1 Serum lipid profiles

	TC (mmol/L)	HDL-C (mmol/L)	TG (mmol/L)
C57 BL/6J STD	3.44 ± 0.19	2.37 ± 0.12	0.50 ± 0.13
ApoE (-/-) HFD	24.49 ± 1.71***	1.07 ± 0.20***	1.75 ± 0.22***
ApoE (-/-) HFD 5 % RBEE	19.24 ± 1.63***,##	1.86 ± 0.20*,###	1.13 ± 0.18*,#

TC total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TG* triglycerides, *STD* standard diet, *HFD* high-fat diet, *RBEE* rice bran enzymatic extract

Values are mean ± SEM of 15 animals. Significant differences are indicated by * ($p < 0.05$); *** ($p < 0.001$) versus C57BL/6J; # ($p < 0.05$), ## ($p < 0.01$); ### ($p < 0.001$) versus ApoE−/− HFD

Rice bran enzymatic extract (RBEE)

RBEE was prepared as previously described following a process based on an enzymatic hydrolysis [22]. Briefly, a hydrolytic trypsin- and chymotrypsin-like endoproteases mixture (Bioproteasa LA450, Biocon Española, Spain) was used for the extraction in a bioreactor, pH (8.0) and temperature (60 °C) controlled using the pH-stat method, resulting in a fully water-soluble syrup and rancidity free due to the inactivation of endogenous lipases. Chemical characterization was previously described by Parrado et al. [22]. Briefly, the major component is protein (38.1 %) in the form of peptides and free amino acids as a result of the use of proteases aiming at RB stabilization. The interaction with fat components (30 %) in the extract allows their solubilization. The following composition of mono- and polyunsaturated fatty acids fatty composition was found in RBEE: 42.44 and 35.76 % of fat content, respectively. Other minor nutraceutical components increased in RBEE compared with RB include phytosterols (4084 mg/kg), γ-oryzanol (1260 mg/kg), tocotrienols (174 mg/kg) and tocopherols (99 mg/kg) [22].

Determination of serum lipids

Blood was collected by intracardiac punction at killing and centrifugated (20 min, 4000 g, room temperature). Serum concentrations of total cholesterol (TC, #Ref.: 1001090, Spin React, CIMA Diagnostics, Girona, Spain), high-density lipoprotein cholesterol (HDL-C, #Ref.: 1001097, Spin React, CIMA Diagnostics, Girona, Spain) and triglycerides (TG, #Ref.: 290-63701, WAKO Diagnostics, Richmond, VA, USA) were determined by enzymatic colorimetric methods using commercial available kits, according to the manufacturer's protocols.

Real-time PCR quantification of telomere length

Aortic and MNC telomere length was quantified using the real-time PCR method established by Cawthon et al. [23] and adapted for mice as previously described [6]. Total DNA was extracted from thoracic aorta and spleen MNC with QIAamp DNA Mini Kits (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. Real-time polymerase chain amplification was carried out in a volume of 25 µl reaction mix (*Power* SYBR® Green PCR Master Mix, Life technologies, Warrington, UK) in a StepOnePlus™ Real-Time PCR System (Life technologies, Thermo Scientific). The primers used were custom-made by Eurofins Genomics (Ebersberg, Germany) and had the following sequences: telomere Fw 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′; telomere Rev 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′, 36b4 Fw 5′-CACACTCCATCATCAATGGGTACAA-3′ and 36b4 Rev 5′-CAGCAAGTGGGAAGGTGTACTCA-3′. PCRs were set up as follows: One initial step at 95 °C for 10 min was followed by 40 cycles consisting of 15 s at 95 °C (denaturation) and 1 min at 58 °C (annealing/extension). In each PCR run, a five-point standard curve to test PCR efficiency was run for both telomere and 36b4 using a DNA pool created from mice of all experimental groups. The cycle threshold (Ct) value differences between telomere DNA and 36b4 DNA for each sample were calculated using the $2^{-\Delta\Delta C_t}$ method as previously described [5].

Telomerase repeat amplification protocol (TRAP)

Telomerase activity was determined using the telomerase repeat amplification protocol (TRAP) assay adapted from the protocol of Kim et al. [24] and as previously described [5]. Aortas and spleen MNC were homogenized in 1X CHAPS buffer (TRAPeze, Millipore, Darmstadt, Germany). Primers were synthesized by Eurofins Genomics (Ebersberg, Germany), and the following sequences were used: TS 5′-AATCCGTCGAGCAGAGGT-3′; ACX 5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′. The reactions, containing 1 µg of protein (aorta) or 10,000 cells (MNC), 0.2 µg TS and 0.1 µg ACX primers were performed in a total volume of 20 µl using Light Cycler® FastStart DNA Master SYBR Green 1 (Roche, Mannheim, Germany). Protein extracts from human embryonic kidney HEK293 cells were used as positive controls in each assay. After incubation at 30 °C for 30 min for template elongation by telomerase activity, real-time PCR was performed in a Roche LightCycler II Thermal Cycler PCR system with 40 cycles of 95 °C for 20 s, 60 °C for 30 s and 72 °C for 50 s after an initial denaturation step. Ct values were determined, and relative telomerase activity was calculated and expressed as HEK293 cell equivalents.

TERT mRNA expression

Expression of TERT was determined by quantitative reverse transcription polymerase chain reaction (*qRT-PCR*). Total RNA was extracted from frozen thoracic aorta and spleen MNC in 1 mL of peqGOLD RNApure (Peqlab, Erlangen, Germany), according to the manufacturer's instructions. Total RNA concentration was determined by measuring absorbance at 260 and 280 nm (NanoDrop 2000, Thermo Scientific). Purity of isolated RNA was assessed by documenting 260/280 nm ratios. Two micrograms of RNA was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life technologies, Foster City, USA) in a peqSTAR 96X Thermal Cycler system (Peqlab, Erlangen, Germany).

The generated cDNA was then amplified with the following primers (Eurofins Genomics, Ebersberg, Germany): TERT Fw 5′-TCCTGAGTATGGGTGCATGA-3′; TERT Rev 5′-AGTGAGCAGGCAGCTGGTAT-3′ and 18 s Fw 5′-TTGATTAAGTCCCTGCCCTTTGT-3′; 18 s Rev 5′-CGATCCGAGGGCCTCACTA-3′ as housekeeping control mRNA. Real-time polymerase chain amplification was carried out in a reaction volume of 20 µl reaction mix (*Power* SYBR® Green PCR Master Mix, Life technologies, Thermo Scientific) in a StepOnePlus™ Real-Time PCR System (Life technologies, Warrington, UK). Briefly, PCRs were set up as follows: The initial denaturation step at 95 °C for 10 min was followed by 40 cycles consisting of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing/extension). Analyses of expression differences between groups were accomplished using the using the $2^{-\Delta\Delta C_t}$ method.

Western blotting

Aortas and spleen MNC were homogenized by sonication in lysis buffer containing 100 mM Tris (pH 6.8), 4 % sodium dodecyl sulfate (SDS), 20 % glycerol, 1 µg/mL leupeptin, 1 µg/mL aprotinin and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) as previously described [5]. Samples were cooked at 90 °C for 5 min to enhance homogenization as described by Werner et al. [5]. After centrifugation at 4000 rpm for 5 min, protein concentrations were determined by Lowry assay. Fifty micrograms of protein was resolved on 10–12 % SDS-polyacrylamide gels. Then, the proteins were blotted onto nitrocellulose membranes and blocked at room temperature for 30 min using dry milk. The membranes were incubated at 4 °C overnight with one of the following primary antibodies: TRF2 (9F10), p53 (D-11), p16 (F-12), bcl-2 (N-19) or bax (B-9) (1:500, Santa Cruz Biotechnology, Heidelberg, Germany). Membranes were then exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4000) or goat anti-mouse IgG (1:5000). Immune complexes were visualized with the use of enhanced chemiluminescence (ECL) and exposition to autoradiography films (GE Healthcare, Munich, Germany), which were developed in an Agfa Curix 60 system. Densitometric analyses were performed using Image Studio Lite v.4.0.21 software (LI-COR, Nebraska, USA), and GAPDH was used as internal control to normalize protein loading in all blots.

Determination of endothelial apoptosis by in situ hairpin oligo ligation assay

In situ aortic endothelial apoptosis was visualized using the ApopTag Peroxidase In Situ Oligo Ligation Apoptosis Detection Kit (Millipore, Billerica, USA) according to the manufacturer's instructions as described before [5, 6, 25]. In brief, the reaction relies on the specificity of the enzyme T4 DNA ligase to join duplex blunt ends of DNA molecules followed by the subsequent formation of a biotinylated oligo hairpin. The thoracic aorta was fixed for 1 h with paraformaldehyde and paraffin-embedded. Five-micrometer sections were deparaffinized and rehydrated with graded ethanol. Endogenous peroxidase was quenched by immersion in 3 % H_2O_2 for 15 min and oligo DNA ligase incubated overnight inside a humidified chamber at 16–23 °C. On the next day, the slides were incubated with streptavidin–horseradish peroxidase solution for 45 min at RT, and 3,3′ diaminobenzidine was added to develop the slides under light microscopic control of the brown staining.

Histological analysis of atherosclerotic lesions and macrophage infiltration

For atherosclerotic lesion analysis, 4 % paraformaldehyde-fixed hearts including the ascending aorta were embedded in Tissue-Tek for cryosectioning. Macrophage infiltration was visualized by immunohistochemistry staining for MAC3+ (1:200, eBioscience, San Diego, CA, USA) with all images recorded using an Olympus BX61 microscope. Macrophage infiltration and atherosclerotic lesions were measured in 10-µm transverse sections of the aortic sinus, and averages were calculated from three serial sections with the help of ImageJ v1.45 software (NIH, USA).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM), and "*n*" reflects the number of assays conducted. Means were compared using ANOVA, followed by Bonferroni's test for multiple comparisons. Statistical comparisons were performed using GraphPad Prism Software v5.01 (San Diego, CA, USA). Differences were considered statistically significant at a p value of <0.05.

Results

RBEE improves serum lipid profiles

High-cholesterol, high-fat diet (HFD) in ApoE−/− mice led to elevated concentrations of serum TC ($p < 0.001$) and TG ($p < 0.001$) and lower concentrations of HDL-C ($p < 0.001$), compared to C57BL/6J. Dietary supplementation with RBEE partially counteracted HFD-induced dyslipidemia causing a 21.4 ± 2.4 % reduction in TC ($p < 0.01$), a 37.7 ± 2.8 % reduction in TG ($p < 0.01$) and a 97.1 ± 9.9 % increase in HDL-C ($p < 0.001$; Table 1).

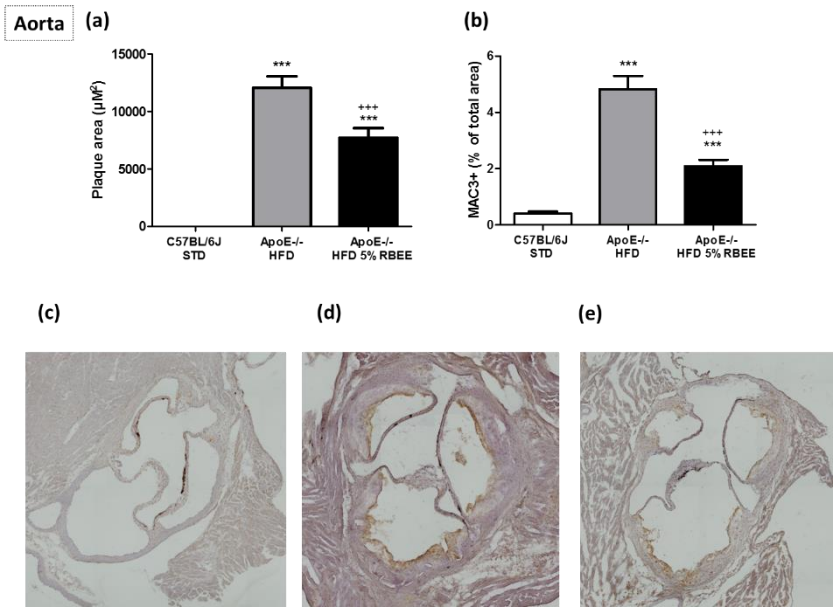


Fig. 1 RBEE attenuates macrophage infiltration and atherosclerotic plaque development. Ten-micrometer aortic sinus sections were used for plaque area and macrophage infiltration determination. **a** Quantification of plaque burden and **b** macrophage infiltration indicated by *brown* precipitate. Representative images of immunostaining for MAC3+ of **c** C57BL/6J, **d** ApoE−/− HFD and **e** ApoE−/− HFD 5 % RBEE groups. Values were mean ± SEM of 7 (C57BL/6J)–8 (ApoE−/−) animals. Significant differences are indicated by ***($p < 0.001$) versus C57BL/6J and by $^{+++}$($p < 0.001$) versus ApoE−/− HFD

RBEE attenuates atherosclerotic plaques and macrophage infiltration

HFD for 23 weeks in ApoE−/− mice resulted in marked aortic atherosclerotic plaque growth (12,040 ± 1001 μm^2 of total aortic area). Macrophage infiltration was increased (MAC3 + area 4.83 ± 0.47 % of total aortic area; $p < 0.001$ vs C57BL/6J, Fig. 1a, b). Dietary supplementation with RBEE potently reduced plaque area in the aortic sinus (7737 ± 836 μm^2 of total aortic area; $p < 0.001$ vs ApoE−/− and $p < 0.001$ vs C57BL/6J, Fig. 1a). In addition, a marked decrease in macrophage infiltration in atherosclerotic plaques of RBEE-treated mice was observed (MAC3 + area 2.09 ± 0.22 % of total aortic area; $p < 0.001$ vs ApoE−/− and $p < 0.001$ vs C57BL/6J, Fig. 1b).

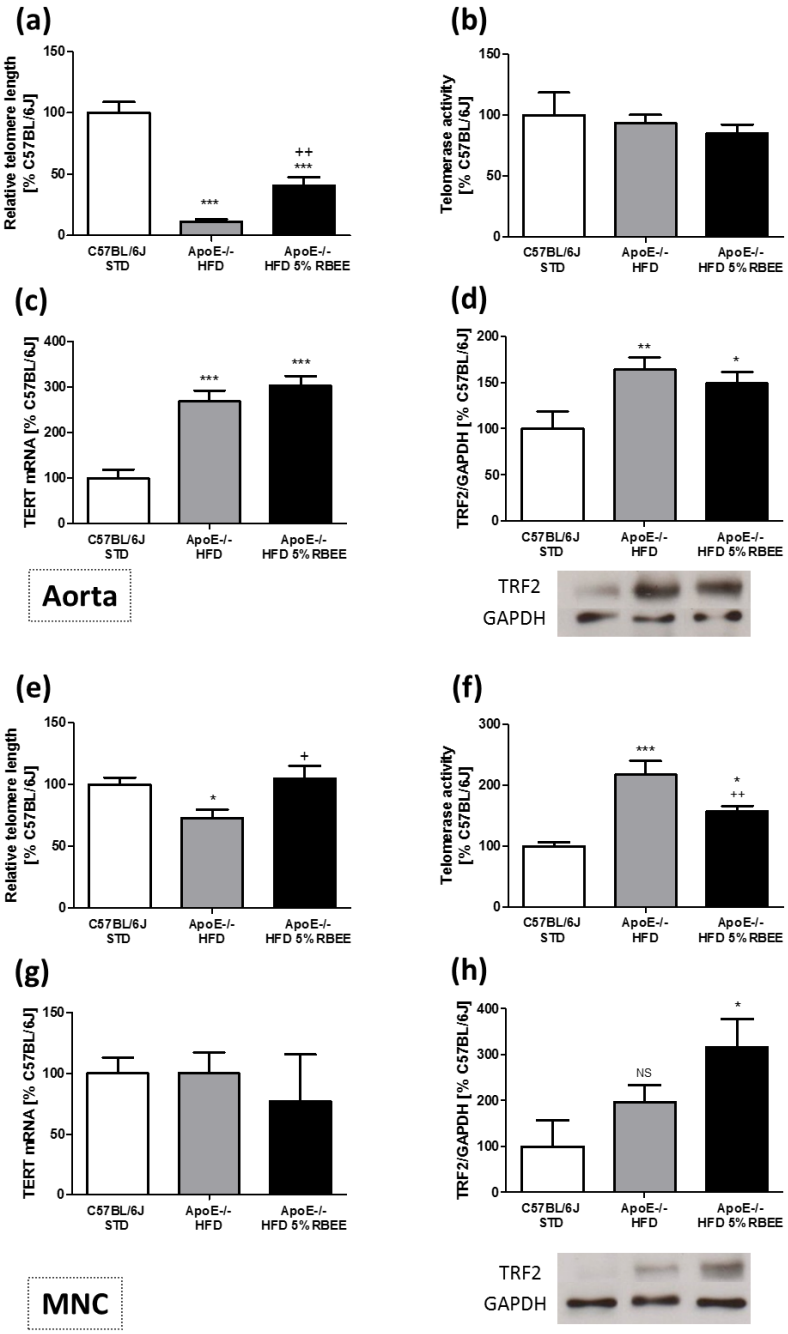
Dietary supplementation with RBEE prevents telomere shortening

ApoE−/− mice fed HFD exhibited shorter telomeres in aorta (11.07 ± 2.34 %, $p < 0.001$, Fig. 2a) and MNC (72.99 ± 6.52, $p < 0.05$, Fig. 2e) compared to C57BL/6J mice. RBEE supplementation was associated with a reduction in telomere erosion, showing a relative telomere length of 40.43 ± 7.42 % ($p < 0.01$ vs ApoE−/− HFD and $p < 0.001$ vs C57BL/6J) in the aorta and 105.0 ± 10.47 ($p < 0.05$ vs ApoE−/− HFD) in spleen MNC compared to C57BL/6J mice.

Telomerase activity, TERT and telomere repeat-binding factor 2 expression

Telomere shortening is regulated by telomerase activity and telomere sheltering proteins such as telomere repeat-binding factor 2 (TRF2), which regulates T-loop formation, telomere capping and therefore protection of telomeres from degradation. Therefore, telomerase activity, the gene expression of its catalytic subunit telomerase reverse transcriptase (TERT) and TRF2 protein expression were assessed.

With respect to telomerase activity, no differences were observed between the groups in the aortic tissue (Fig. 2b). However, TERT mRNA was more abundant in both ApoE−/− groups ($p < 0.001$ ApoE−/− HFD vs C57BL/6J,


(a)
Relative telomere length [% C57BL/6J]
150
100
50
0
***
++
***
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
(b)
Telomerase activity [% C57BL/6J]
150
100
50
0
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
(c)
TERT mRNA [% C57BL/6J]
400
300
200
100
0
***
***
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
Aorta
(d)
TRF2/GAPDH [% C57BL/6J]
200
150
100
50
0
**
*
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
TRF2
GAPDH
(e)
Relative telomere length [% C57BL/6J]
150
100
50
0
*
+
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
(f)
Telomerase activity [% C57BL/6J]
300
200
100
0
***
*
++
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
(g)
TERT mRNA [% C57BL/6J]
150
100
50
0
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
MNC
(h)
TRF2/GAPDH [% C57BL/6J]
400
300
200
100
0
NS
*
C57BL/6J STD
ApoE-/- HFD
ApoE-/- HFD 5% RBEE
TRF2
GAPDH

◀**Fig. 2** Telomere length, telomerase activity and TRF2 expression in aorta and spleen-derived MNC. Relative telomere length expressed as percentage of C57BL/6J was measured by quantitative real-time PCR in aorta (**a**) and spleen MNC (**e**). Telomerase activity was measured in aorta (**b**) and spleen MNC (**f**) using 1 μg of protein (aorta) or 10,000 cells (MNC) by telomerase repeat amplification protocol (TRAP assay). Telomere and single copy gene 36b4 standard curves were derived using serial dilutions of a DNA pool. Telomerase reverse transcriptase (TERT) mRNA levels (normalized to 18 s) were determined by quantitative real-time PCR in aorta (**c**) and spleen MNC (**g**). Telomere repeat-binding factor (TRF) 2 expression (normalized to GAPDH) was quantified by Western blot of aortic tissue (**d**) and spleen MNC homogenates (**h**). C57BL/6J mice were used as the comparator group, and % values are mean ± SEM of 5 (C57BL/6J)–6 (ApoE−/−) animals. Significant differences are indicated by *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$) versus C57BL/6J and by $^{+}$($p < 0.05$) and $^{++}$($p < 0.01$) versus ApoE−/− HFD

Fig. 2c). Similarly, TRF2 protein expression was increased compared to WT in the aorta ($p < 0.01$ ApoE−/− HFD vs C57BL/6J and $p < 0.05$ ApoE−/− HFD 5 % RBEE vs C57BL/6J, Fig. 2d), showing two possible mechanisms for telomere protection.

Regulation of telomere-associated proteins was different in MNC compared to aorta. Telomerase activity was enhanced by 218 ± 23 % in ApoE−/− MNC ($p < 0.001$, Fig. 2f). This increase was reduced to 158 ± 8 % by RBEE supplementation ($p < 0.05$ vs C57BL/6J and $p < 0.01$ vs ApoE−/− HFD), lending support to the concept that in MNC up-regulation of telomerase activity may be compensatory to HFD-induced telomere damage. This increment could not be explained by an up-regulation of TERT, which was comparable between groups (Fig. 3g). However, RBEE supplementation led to an up-regulation of TRF2 expression by 317 ± 61 % ($p < 0.05$ vs C57BL/6J, Fig. 2h).

Treatment with RBEE exerts anti-apoptotic effects in the aorta

Apoptosis in atherosclerotic lesions is triggered by DNA damage and inflammatory processes induced by high cytokine and oxidized lipids levels [26]. Apoptosis was evaluated in the aorta through the use of two independent assays.

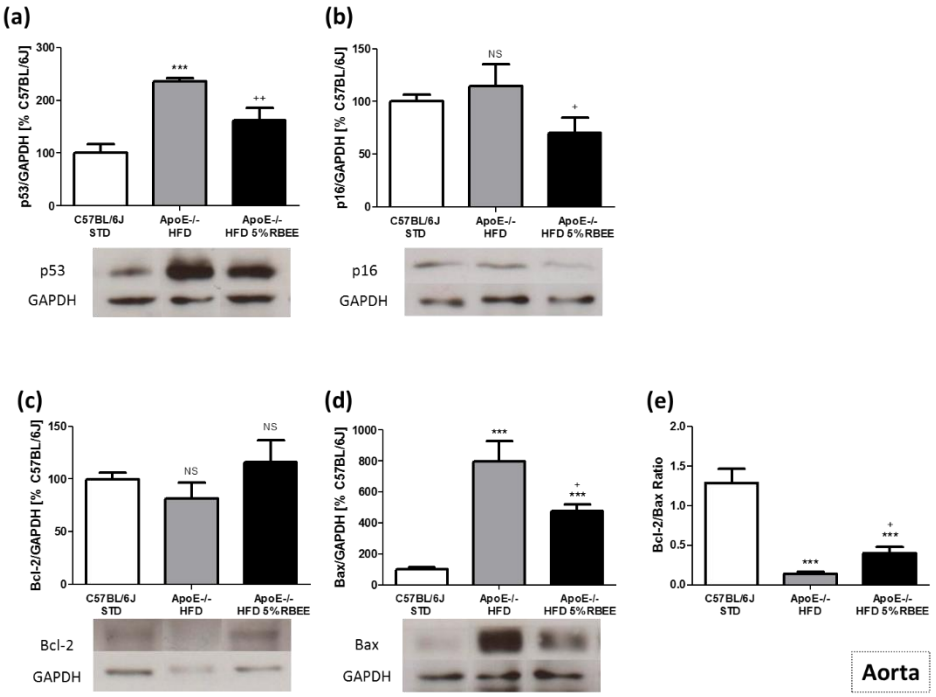


Fig. 3 Apoptosis and senescence markers in the aorta. Aortic protein expression of apoptosis and senescence markers was determined by Western blots **a** p53, **b** p16, **c** bcl-2, **d** bax and **e** bcl-2/bax ratio in C57BL/6J, ApoE−/− HFD and ApoE−/− HFD 5 % RBEE groups. GAPDH was used as housekeeping protein. Values were mean ± SEM of 5 (C57BL/6J)–6 (ApoE−/−) animals. Significant differences are indicated by ***($p < 0.001$) versus C57BL/6J and by $^{+}$($p < 0.05$) and $^{++}$($p < 0.01$) versus ApoE−/− HFD

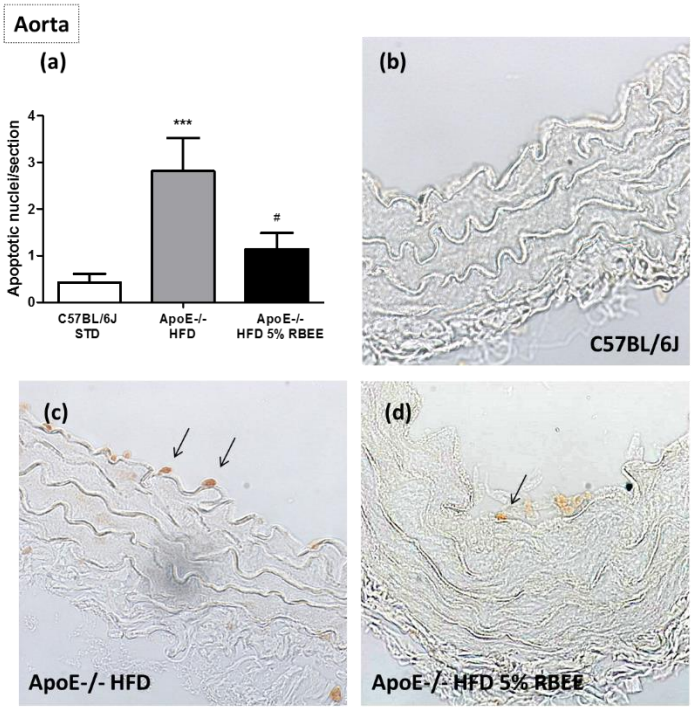


Fig. 4 Histological determination of endothelial apoptosis. Five-micrometer paraffin-embedded sections of the thoracic aorta were used for in situ determination of endothelial apoptosis. Arrows show positive apoptotic nuclei. **a** Quantification of apoptotic nuclei per section. Representative sections of **b** C57BL/6J, **c** ApoE−/− HFD and **d** ApoE−/− HFD 5 % RBEE groups. RBEE reduced the number of apoptotic nuclei per ring from 2.81 ± 0.71 to 1.14 ± 0.35 ($p < 0.05$ vs ApoE−/− HFD). Values are mean ± SEM of 8 (C57BL/6J)–9 (ApoE−/−) animals

First, protein expression of components of the apoptosis cascade was assessed by Western blots (WB), which revealed increased levels of p53 ($p < 0.001$, Fig. 3a) and bax ($p < 0.001$, Fig. 3d) and strongly reduced bcl-2-to-bax ratio ($p < 0.001$, Fig. 3e) in ApoE−/− HFD compared to C57BL/6J control mice. Supplementation with RBEE reduced p53 up-regulation by 30 ± 5 % ($p < 0.01$ vs ApoE−/− HFD, Fig. 3a). Expression of the cell cycle inhibitor/senescence marker p16 was reduced by 39 ± 5 % ($p < 0.05$ vs ApoE−/− HFD, Fig. 3b) by RBEE. The mitochondrial apoptosis regulator bax was also down-regulated (40 ± 4 %; $p < 0.05$ vs ApoE−/− HFD and $p < 0.01$ vs C57BL/6J, Fig. 3d), resulting in a relative increase in bcl-2/bax ratio by 249 ± 1 % in RBEE-treated mice ($p < 0.05$ vs ApoE−/− HFD and $p < 0.001$ vs C57BL/6J, Fig. 3e).

Secondly, to directly confirm apoptosis prevention histologically, hairpin assays were performed in paraffin-embedded aortas (Fig. 4). In correlation with apoptosis marker expression measured by WB, supplementation with RBEE reduced the number of apoptotic nuclei per ring from 2.81 ± 0.71 to 1.14 ± 0.35 ($p < 0.05$ vs ApoE−/− HFD, Fig. 4a).

RBEE enhances HFD-induced p53, bax and bcl-2 expression in MNC

Inflammatory cell infiltration into the aortic tissue was reduced in RBEE-treated ApoE−/− mice on HFD (see above). Therefore, next we measured the effects of RBEE on expression of apoptosis and senescence regulators in spleen-derived MNC (Fig. 5).

Up-regulation of p53 protein expression observed in HFD-fed mice ($p < 0.05$, Fig. 5a) was enhanced by RBEE treatment by 74 ± 12 % ($p < 0.05$ vs ApoE−/− HFD and $p < 0.001$ vs C57BL/6J, Fig. 5a). In parallel, the p53-downstream mediator bax showed an increased expression (176 ± 33 %; $p < 0.01$ vs ApoE−/− HFD and $p < 0.05$ vs C57BL/6J). However, this raise in bax expression was paralleled by an up-regulation of the anti-apoptotic regulator bcl-2 (202.11 ± 13.84 %; $p < 0.001$ vs ApoE−/− HFD and $p < 0.001$ vs C57BL/6, Fig. 5d), leading to an unchanged bcl-2/bax ratio in RBEE-treated HFD-ApoE−/− mice (Fig. 5e).

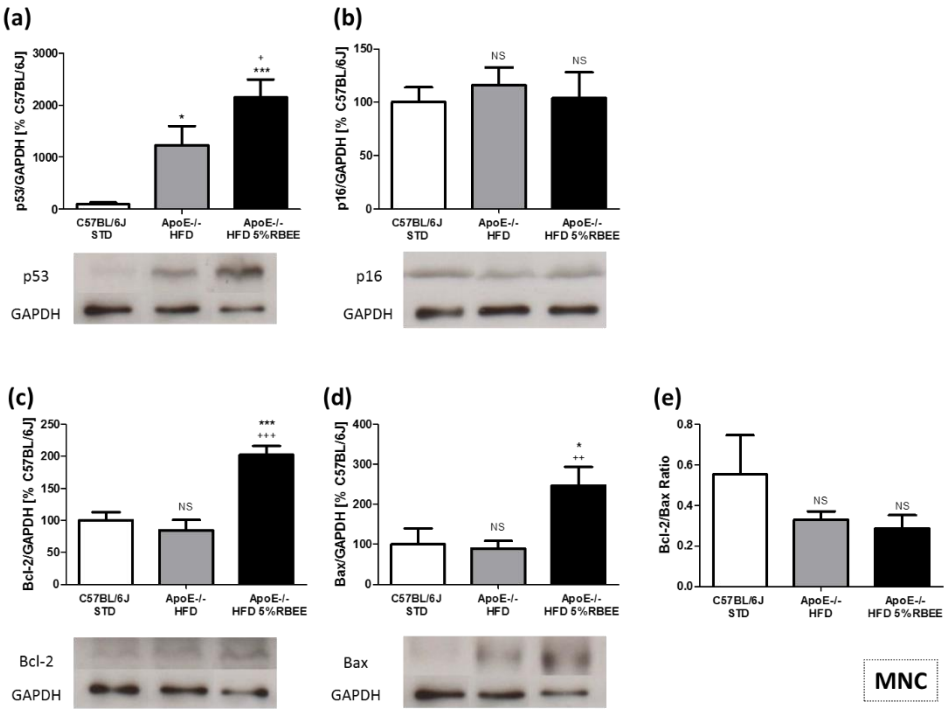


Fig. 5 Apoptosis and senescence markers in spleen MNC. Western blots showing MNC expression of **a** p53, **b** p16, **c** bcl-2, **d** bax and **e** bcl-2/bax ratio in C57BL/6J, ApoE−/− HFD and ApoE−/− HFD 5 % RBEE groups. GAPDH was used as housekeeping protein. Values were mean ± SEM of 5 (C57BL/6J)–6 (ApoE−/−) animals. Significant differences are indicated by $^{*}(p < 0.05)$ and $^{***}(p < 0.001)$ versus C57BL/6J and by $^{+}(p < 0.05)$, $^{++}(p < 0.01)$ and $^{+++}(p < 0.001)$ versus ApoE−/− HFD

Discussion

Rice bran is rich in γ-oryzanol, tocopherols and phytosterols and has been reported to exert antioxidant, lipid-lowering and anti-inflammatory effects [18–21]. We have developed a novel soluble non-rancid syrup preparation of rice bran with enriched content in tocols and γ-oryzanol [22]. The main novel finding of this study is that a diet supplemented with this rice bran extract (RBEE) protects against cellular senescence and apoptosis in the vascular wall and potently prevents the development of atherosclerotic plaques in mice. These effects were associated with improved serum lipid profiles and reduced telomere shortening in the vessel wall and in spleen-derived MNC.

The apolipoprotein E-deficient mouse is the most widespread model for atherosclerosis research from several in vivo models available. Its spontaneous propensity to rapid serum cholesterol accumulation and plaque formation, similar to the concept of atherosclerosis in humans, both on regular chow and to much more extent when consuming a high-fat, high-cholesterol diet (HFD) makes ApoE−/− mice a suitable and reliable model for atherosclerosis research. Here, supplementation of HFD with 5 % RBEE resulted in a marked improvement of plasma lipid profiles. Previous works demonstrated RBEE's antioxidant, lipid-lowering and anti-inflammatory potential among other obesity-related vascular complications in Wistar rats and in a Zucker rat model of metabolic syndrome [27–31]. The data agree with numerous studies reporting lipid-lowering effects of micronutrients of RBEE as part of rice bran oils or rice bran enzymatic extracts in animals and humans through mechanisms involving HMG-CoA reductase inhibition [32, 33] and decreased cholesterol absorption [34–36]. These are the main mechanisms exploited by lipid-lowering therapies in humans.

Aging is an independent risk factor for atherosclerosis [37]. Aged vessels, even in the absence of atherosclerotic plaques, undergo remodeling leading to cardiovascular

diseases. On the other hand, atherosclerosis involves increased turnover rate promoting cellular senescence. In the same sense, an inverse relation has been established between MNC telomere length and atherosclerosis progression [38]. The data here presented show telomere protection of RBEE in aorta and MNC not dependent on telomerase activity. In fact, RBEE diet supplementation reduced telomerase activity in ApoE−/− MNC, but induced telomeric repeat-binding factor 2 (TRF2) expression, suggesting better telomere capping as a possible protection against telomere erosion. The anti-telomerase activity may be triggered by the tocotrienols present in RBEE, via protein kinase C inhibition, which has been shown to down-regulate c-Myc and telomerase reverse transcriptase (TERT) expression in other models [39, 40]. In tissues with inflammatory stimuli, such as atherosclerosis, TERT can be transiently up-regulated by NF-κβ signaling pathway [41] as we observed in the aorta of ApoE−/− mice. However, since telomerase activity was unchanged in aortic lysates, the observed protection from HFD-induced telomere erosion by RBEE must be explained by other effects. Oxidative stress accelerates erosion and loss of telomeric DNA [42]. RBEE is rich in antioxidant components such as γ-oryzanol, ferulic acid, tocols and sulfur amino acids (cysteine and methionine), which are precursors of the antioxidants taurine and glutathione [22, 27]. Previous data demonstrated RBEE's capacity of avoiding lipid peroxidation and protein oxidation in vitro and ex vivo through scavenging hydroxyl and peroxyl radicals [43, 44].

In atherosclerotic lesions, macrophage accumulation correlates with plaque development by accumulation of cholesterol and fatty acids. Therefore, macrophage apoptosis may have a positive effect in terms of stability and progression of the atherosclerotic lesions [45, 46]. Here, increased apoptosis of spleen MNC induced by RBEE-supplemented diet may disrupt macrophage development, leading to lower infiltration and plaque growth. Augmented apoptosis of spleen MNC, as revealed by increased expression of tumor suppressor protein p53 and its downstream effector bax, may be induced as previously described by several RBEE components. γ-oryzanol, ferulic acid, β-sitosterol and specially tocopherols/tocotrienols have been found to induce apoptosis and inhibit cell growth in different cell lines of cancer while having low or no effect in non-malignant cells [47]. In contrast, we observed that RBEE induced a down-regulation of tumor suppressor proteins p53, p16 and bax along with an increase in bcl-2-to-bax ratio in the aorta. Oxidative and pro-inflammatory status induced by HFD may cause DNA injury activating cell death through apoptosis. RBEE-mediated alleviation of this pro-apoptotic environment as well as DNA protection may explain the down-regulation of the p53 signaling pathway. Previous works demonstrated the protective role of α-tocopherol and γ-tocopherol in oxLDL-induced apoptosis in human coronary smooth muscle cells by up-regulation of bcl-2, which blocks cell death, and by reducing kinase cascade activation (MAPK and JunK) [48]. Another component of RBEE, ferulic acid, was found to inhibit apoptosis of peripheral blood cells by a bcl-2-independent mechanism related to antioxidant activities and reduction in DNA damage [49].

Interestingly, RBEE had a dual and opposite effect on MNC and aorta. RBEE provided remarkable telomere protection in MNC and aorta, but enhanced apoptosis in MNC, and at the same time apoptosis was reduced in the aorta. Such differences could be attributed to one of the components of RBEE, namely tocopherols, which have been found to display pro-apoptotic and anti-proliferative effects upon cells with a high turnover such as in carcinogenic tissues while having low or no effect in non-malignant cells [47]. This dual effect may be a novel mechanism for the observed reduction in macrophage infiltration and plaque development. However, the specific mechanism of RBEE for this dual effect or whether it is the sole cause for plaque reduction remain to be further studied. RBEE reduced telomere attrition in both MNC and aortic tissue by means independent of telomerase activity. These effects should be further clarified in future investigations. This study was limited by the lack of knowledge of the individual contribution of each active component of RBEE for the different activities here described and its actual bioavailability.

In conclusion, food supplementation with rice bran enzymatic extract prevented HFD-associated telomere shortening in aorta and MNC and reduced apoptosis of vascular endothelial cells in ApoE−/− mice. Enhanced apoptosis of spleen MNC along with improved serum lipid profiles may explain reduced macrophage infiltration and plaque development observed in RBEE-treated mice demonstrating the potential of RBEE diet supplementation in high-fat-induced vascular disease due to its antioxidant, lipid-lowering and anti-inflammatory properties. Future trials must now elucidate whether RBEE exerts similar effects in humans with lipid disorders and vascular disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards Approval for all experiments was provided by the Ethic Committee for Animal Experimentation of the University of

Seville (Spain) (AGL2013-407791-P). The manuscript does not contain clinical studies or patient data.

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Supplementary table 1: Diets and RBEE nutritional composition. Energy content and dietary macronutrients of standard (STD), high fat (HFD), and high fat supplemented (HFD 5% RBEE) diets and rice bran enzymatic extract (RBEE).

Nutrient	STD	HFD	HFD 5% RBEE	RBEE
Energy (Kcal/g)	2.90	4.50	4.52	4.83
Protein (% Kcal)	20.0	15.2	16.0	31.7
Carbohydrate (% Kcal)	67.0	42.7	41.2	11.7
Fat (% Kcal)	13.0	42.0	42.7	56.7

CAPÍTULO VI

Reunirse es un comienzo, permanecer juntos es un progreso, trabajar juntos es el éxito

(Henry Ford, 1863-1947)

BIODISPONIBILIDAD DE LOS COMPUESTOS FENÓLICOS DEL EXTRACTO ENZIMÁTICO DE SALVADO DE ARROZ. ACTIVIDAD SOBRE LA PRODUCCIÓN DE ANIÓN SUPERÓXIDO

Perez-Ternero C, Macià A, Parrado J, Alvarez de Sotomayor M, Herrera MD, Motilva MJ

En revisión en *Molecular Nutrition & Food Research*

Existen varios estudios que han tratado de determinar sin éxito la biodisponibilidad por vía oral del γ-oryzanol, uno de los principales componentes bioactivos del salvado de arroz. El γ-oryzanol es una molécula compuesta por una mezcla de esteres del ácido ferúlico con alcoholes triterpénicos o fitosteroles. El enlace éster puede ser hidrolizado mediante esterasas en el proceso de la digestión, liberando al ácido ferúlico para que sea absorbido mientras que la mitad del alcohol triterpénico o fitosterol quedaría en el intestino contribuyendo a la actividad hipolipemiante, entre otras. Como objetivo de este trabajo se planteó el estudio de la biodisponibilidad oral y ruta metabólica de los compuestos fenólicos presentes en el extracto enzimático de salvado de arroz (EESA) y el efecto de los mismos sobre la producción de anión superóxido.

Ratas Wistar de 12 semanas de edad fueros sondadas con 10 g EESA/kg peso y sacrificadas 0 min, 15 min, 30 min, 60 min, 3 h, 6 h, 12 h, 18 h y 24 h después de la administración del EESA para la extracción de suero. Otro grupo de ratas fueron empleadas para la recogida de heces y orina a diferentes tiempos durante 48 h. Los metabolitos del ácido ferúlico presentes en suero, heces y orina fueron determinados mediante UHPLC-MS/MS. Adicionalmente, la producción de anión superóxido inducida con endotelina 1 (ET-1, 10 nM), fue determinada en anillos de aorta en presencia de EESA, ácido ferúlico o γ-oryzanol o del suero de ratas sondadas con EESA.

De un total de 25 metabolitos encontrados en plasma, el ácido dihidroxifenilacetico sulfato fue el mayoritario, encontrándose su pico de absorción (C_{max}) a los 30 min. Se observaron diferentes cinéticas de absorción con comportamientos bifásicos, que mostraron C_{max} a diferentes tiempos. Los metabolitos encontrados en plasma también fueron detectados en orina, siendo la excreción mayor en el periodo comprendido entre las 24 y las 48 h. En heces, la mayoría de los compuestos encontrados fueron metabolitos de fase II sin conjugar, lo que indica la participación del metabolismo bacteriano.

La incubación de los anillos de aorta con ácido ferúlico a la concentración encontrada en el suero, o la cantidad equivalente de EESA o γ-oryzanol, o con el suero de las ratas tratadas con EESA, redujo la producción de anión superóxido inducida por ET-1.

Con todo esto, podemos concluir que la molécula de γ-oryzanol, presente en el EESA, sufre una hidrólisis durante el proceso de digestión, liberando al ácido ferúlico que es rápidamente absorbido (t_{max}: 27.00 ± 3.00 min) y queda disponible en el plasma desde donde ejerce propiedades antioxidantes sobre la actividad de la NADPH oxidasa a nivel vascular.

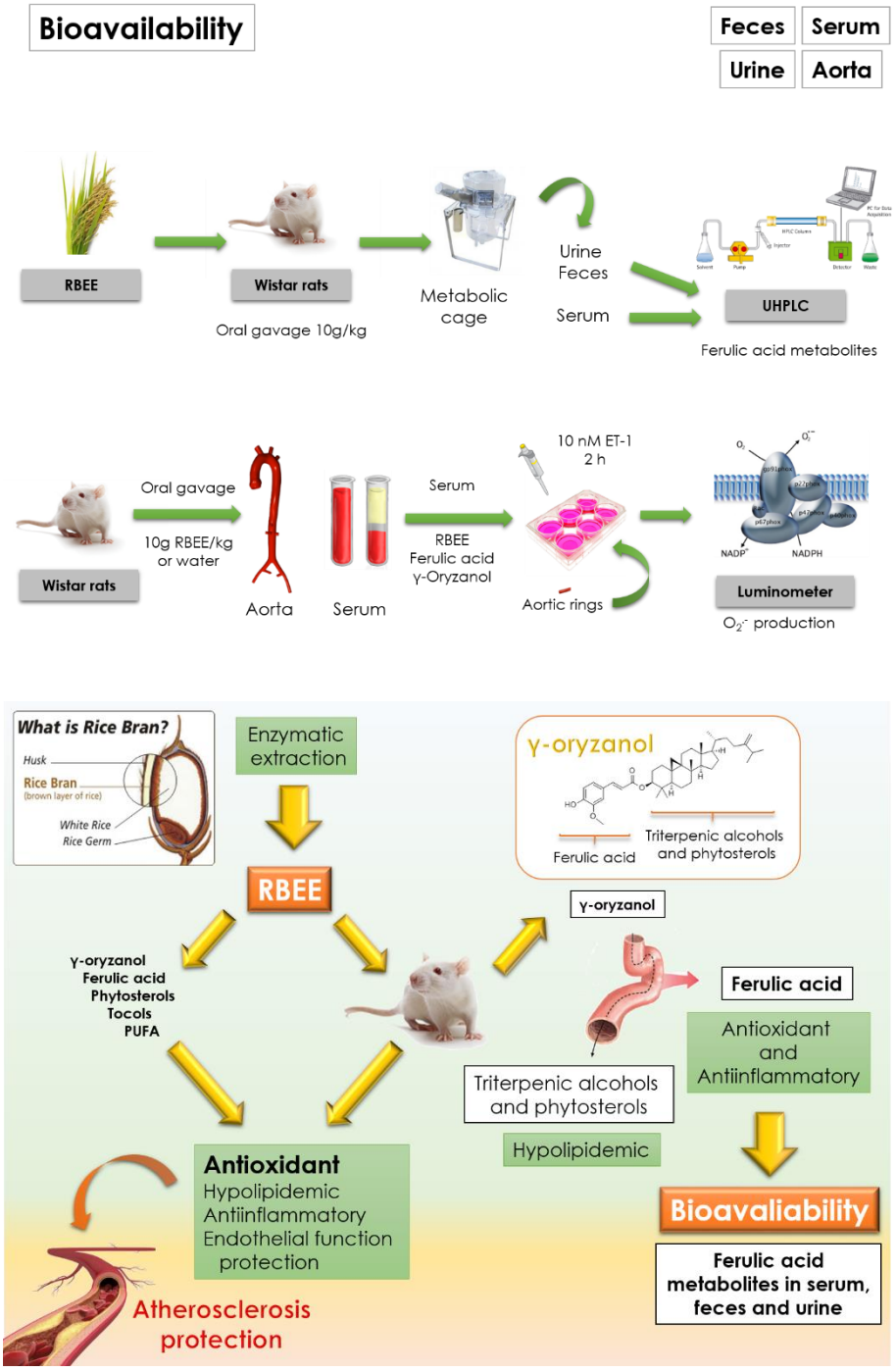

Figura 20: Diseño experimental y resumen de resultados.

BIOAVAILABILITY OF THE FERULIC ACID-DERIVED PHENOLIC COMPOUNDS OF A RICE BRAN ENZYMATIC EXTRACT. ACTIVITY AGAINST THE SUPEROXIDE PRODUCTION

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Abstract

Scope: Rice bran is an exceptional source of such antioxidant molecules as γ-oryzanol and ferulic acid, but their bioavailability and metabolism remain unknown. The aims of this work were to describe the oral bioavailability and metabolic pathways of the ferulic acid-derived phenolic compounds contained in a rice bran enzymatic extract (RBEE), and to determine its effect on NADPH oxidase activity.

Methods and results: Wistar rats were administrated with RBEE and sacrificed at different times over a period of 24h to obtain plasma. Urine and faeces were collected for 48h. The phenolic metabolites were determined by liquid chromatography (UHPLC-MS/MS), and plasma pharmacokinetic parameters were calculated. In parallel, aortic rings were incubated in the plasma of rats sacrificed 30 min after RBEE gavage, or in the presence of RBEE, ferulic acid or γ-oryzanol. Endothelin-1-induced superoxide production was recorded by lucigenin-enhanced luminescence.

Conclusion: Twenty-five ferulic acid metabolites showing biphasic behaviour were found in the plasma, most of which were found in the urine as well, while in the faeces, colonic metabolism led to the simple phenol compounds. Superoxide production was abrogated by phenolic compound-enriched plasma and by RBEE and ferulic acid, thus showing the biological potential of RBEE as a nutraceutical ingredient.

Keywords: Ferulic acid; metabolites; bioavailability; NADPH oxidase; rice bran enzymatic extract.

1. Introduction

Rice bran (RB), a by-product of rice milling, is an important source of fat, proteins and bioactive molecules with special interest due to their antioxidant and lipid-lowering properties. These bioactive molecules include γ-oryzanol (ORZ) (a mixture of ferulic acid (FA) esters of triterpene alcohols and sterols), tocols (tocopherols and tocotrienols) and unsaturated fatty acids [1]. RB is especially rich in the phenolic compounds ORZ and FA, which have demonstrated hypolipidemic effects (reducing total plasma cholesterol and triglyceride levels, and increasing high-density lipoprotein levels) by mechanisms related to strong antioxidant activity [2, 3], HMG-CoA inhibition [4] and increased cholesterol excretion [5, 6].

Although RB shows a significant level of natural antioxidants and nutritional proteins [7], its potential use as a functional food ingredient is limited due to the low water solubility of some of its components, including ORZ. These limitations have been overcome by the development of a novel enzymatic extraction process, giving rise to the water-soluble rice bran enzymatic extract (RBEE) [8] used in this study. This extraction method preserves the functional properties and improves the solubility of the proteins and antioxidant components of RB [9, 10], providing significant advantages over other RB-derived products, such as rice bran oil [11]. Moreover, the enzymatic treatment also increases the protein concentration and minor functional components, especially ORZ and tocols.

Over recent years, our group has shown that sustained diet supplementation with RBEE is able to improve cardiometabolic markers in obese Zucker rats and prevent the development of atherosclerosis in ApoE-/- mice [12-14] through the reduction of oxidative stress and inflammation markers in the aorta and mesenteric arteries [13, 14], and in adipose tissue [15-18]. Moreover, RBEE counteracted the deterioration of adipose tissue morphology and the expression of genes related to macrophage polarization in high-fat diet-induced obesity [19]. Interest in RBEE is increasing due to its pharmacological activities, revealing the possibility of using it as a nutraceutical ingredient. Therefore, to explore and determine the mechanisms of action of RBEE bioactive compounds and their role in disease prevention, it is crucial to understand the extent of their absorption and their fate in the organism. Nevertheless, the metabolism and bioavailability of the main phenolic compounds of RBEE remains unknown, this data being essential for any therapeutic use of the extract in humans.

The main objective of this study was to describe the plasma pharmacokinetics of the main ferulic acid-derived compounds biosynthesized after RBEE consumption by pointing out the bioavailability of its main metabolites and the metabolic pathways

involved. We further evaluated the effects of these plasma ferulic acid-derived metabolites on the superoxide production by the NADPH oxidase complex (NADPHox).

2. Material and Methods

2.1 Chemicals and reagents

Standards of catechol, *p*-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(2,4-dihydroxyphenyl)propionic acid, caffeic acid, *p*-coumaric acid and FA were purchased from Sigma-Aldrich (St. Louis, MO, USA), and vanillic acid from Fluka (Buchs, Switzerland). Methanol (HPLC grade), acetone (HPLC grade), glacial acetic acid and hydrochloric acid (HCl) were provided by Scharlau Chemie (Barcelona, Spain). Acetonitrile (HPLC grade) was from Romyl (Teknokroma, Barcelona, Spain). Ortho-phosphoric acid 85% was purchased from Mont Plet & Esteban S.A. (Barcelona, Spain). Water was of Milli-Q quality (Millipore Corp, Bedford, MA, USA).

2.2 Rice bran enzymatic extract (RBEE)

A water-soluble RBEE was prepared as previously described [8] by endoprotease (trypsin- and chymotrypsin like) hydrolysis. The process was conducted in a bioreactor with the pH (pH 8) and temperature (60 °C) controlled. After enzymatic extraction, the nutraceutical composition of the RBEE was characterized as follows: ORZ (8950 ± 850 mg/kg), phytosterols (3553 ± 66 mg/kg), free FA (351 ± 5 mg/kg), tocotrienols (170 ± 15 mg/kg) and tocopherols (93.4 ± 10 mg/kg). The ORZ present in the RBEE was composed of different stearyl ferulate esters (such as 2,4-methylene cycloartenyl ferulate, campesteryl ferulate, cycloartenyl ferulate and sitosteryl ferulate).

2.3 Treatment of rats and plasma, urine and faces collection

Fifty male Wistar rats (body weight 295.8 ± 30.5 g) were purchased from the Central Animal Research Facility of the University of Seville (Espartinas, Seville, Spain). The animals were maintained in groups in an air-conditioned room at 25 °C and 65-70% humidity and with a 12-h light-dark cycle. They were fed a maintenance diet of Harlan (2014, Harlan Laboratories, Madison, USA) and water. All experimental procedures were approved by the University of Seville's (Spain) Committee for Ethical Experimentation (Reference: 12-01-16-002).

At the age of 12 weeks, the rats were randomly assigned to 8 groups (*n=5*) and starved overnight before oral gavage of the equivalent amount of diluted RBEE to 10 g/kg body

weight. Then, the rats were anesthetized with phenobarbital (i.p. 50 mg/kg) and blood was extracted by cardiac puncture at the following endpoints: 0 min, 15 min, 30min, 60 min, 3h, 6h, 12h, 18h and 24h. Plasma was obtained by centrifugation (1500 *g*, 20 min at 4 ºC) and immediately frozen for further determination. An additional group of rats (*n=5*) were housed in individual metabolic cages and used for the collection of urine (0, 1, 2, 3, 4, 5, 6, 9, 24 and 48 h) and faeces (0, 24, 36 and 48 h).

2.4 Chromatographic analysis of ferulic acid–derived metabolites in biological samples

2.4.1. Sample pre-treatment: Before chromatographic analyses, a sample (plasma, urine and faeces) pre-treatment was done in order to extract the phenolic metabolites generated and to remove the interference of the biological matrix. There were three replicates of each biological sample.

Plasma samples. The plasma samples were pre-treated by off-line micro-Elution solid-phase extraction (µSPE) using OASIS HLB (2 mg, Waters, Milford, MA) micro-cartridges. These were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. 350 µL of 4% phosphoric acid was added to 350 µL of the plasma sample, and then this solution was loaded into the micro-cartridges. The loaded micro-cartridges were cleaned-up with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. Then, the retained phenolic compounds were eluted with 2 x 50 µL of the acetone:Milli-Q water:acetic acid (70:29.5:0.5, v/v/v) solution. 2.5 µL of the elution solution was injected into the chromatographic system.

Urine samples. The rat urine samples were also pre-treated by off-line µSPE. 100 µL of 4% phosphoric acid was added to 100 µL of the urine sample before loading into the micro-cartridges. The retained phenolic compounds were then eluted with 2 x 50 µL of the acetone:Milli-Q water:acetic acid (70:29.5:0.5, v/v/v) solution. 2.5 µL of the elution solution was injected into the chromatographic system.

Faeces samples. In order to extract the phenolic metabolites, 0.1 g of lyophilized faeces was mixed in 1 mL of methanol:HCl:Milli-Q water (79.9:0.1:20, v/v/v)and centrifuged (8784 *g*, 5 min at 4 ºC) after 15 min of shaking. The supernatant was collected and re-centrifuged under the same conditions. The resulting supernatant was filtered with nylon filters (0.22 µm filter pore size) and 2.5 µL of this solution was injected into the chromatographic system.

2.4.2 Liquid chromatography analyses (UPLC-ESI-MS/MS)

The phenolic compounds generated were analyzed by AcQuity Ultra-Performance™ liquid chromatography coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford). The analytical column was an AcQuity BEH C_{18} column (100 mm x 2.1 mm i.d., 1.7 µm,) equipped with a VanGuard™ Pre-Column AcQuity BEH C_{18} (2.1 x 5 mm, 1.7 µm), also from Waters. During the analysis, the column was kept at 30ºC and the flow rate was 0.3 mL/min. The mobile phase was 0.2% acetic acid (eluent A), acetonitrile (eluent B). The elution gradient was 0-5 min, 5-10% B; 5-10 min, 10-12.4% B; 10-18 min, 12.4-28% B; 18-23 min, 28-100% B; 23-25.5 min, 100% B isocratic; 25.5-27 min, 100-5% B; and 27-30 min, 5% B isocratic.

Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface (ESI). Ionization was achieved using the electrospray interface operating in the negative mode $[M-H]^-$ and the data was acquired through selected reaction monitoring (SRM). The ionization source parameters were the ones reported in our previous studies [20]. Two SRM transitions were selected, one being the most sensitive transition for quantification, and a second one for confirmation purposes. The SRM transition for quantification and the individual cone voltage and collision energy for each phenolic compound and metabolite are shown in **Supplementary Table 1S**. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx 4.1 software.

2.5. Superoxide production studies

Superoxide anion production was measured in isolated aortic rings by lucigenin-enhanced luminescence, as previously described [21]. Two assays were performed. In the first one, 5-mm aortic rings were incubated with plasma samples from rats sacrificed 30 min after oral gavage of (i) 10 g RBEE/kg body weight (RBEE group), which contained 1.56 ± 0.35 µmol/L FA and different concentrations of its metabolites in plasma 30 min after RBEE intake (**Supplementary Table 2S**), or (ii) the equivalent volume of water (control group, Water) (Section 2.3). In the second assay, the aortic rings were incubated in HEPES-KHS with RBEE (367.6 mg/L), FA (5.5 µmol/L) or ORZ (5.5 µmol/L), respectively. The FA concentration corresponds to the AUC found in the plasma over 24 h after oral gavage and the RBEE concentration is that which contains 5.5 µmol/L FA. Diphenylleneiodininum (DPI, 10 µmol/L) was used as a positive control. Temperature-controlled (37 °C) incubation in the above-described conditions was performed for 2h in the presence of endothelin-1 (ET-1, 10 nmol/L). Then, the rings were transferred to tubes containing 500 µL of HEPES-KHS buffer plus lucigenin (5 µmol/L). Basal relative luminescence units (RLU) were recorded for 3 min. Then, NADPH was added to a final concentration of 100 µmol/L and RLUs were measured every minute for 5 min in a luminometer (Junior LB 9509, Berthhold, Germany). The background and basal

luminescence were subtracted, and the RLUs per minute were normalized to the dry weight of the aortic ring tissue.

2.6 Statistical analysis and pharmacokinetic parameters

Statistical analysis was performed using the Graphpad Prism software v5.01 (San Diego, USA). The data were analysed via analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. Values of *p* lower than 0.05 were regarded as statistically significant. The data are presented as mean values ± standard error of the mean.

The kinetic parameters of the main metabolites of RBEE in the plasma were calculated by means of pharmacokinetic (PK) functions (for Microsoft Excel). The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule method. The peak plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) were the observed values.

3. Results

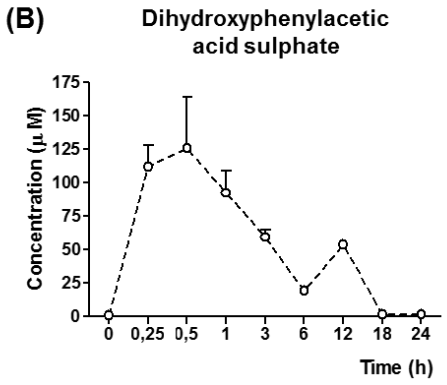
3.1 Determination of ferulic acid-derived phenolic metabolites in plasma

After the acute intake of RBEE, different phenolic metabolites were identified in the plasma, urine and faeces. **Figure 1A** shows the pharmacokinetic parameters, C_{max} (μM), t_{max} (min), and AUC (μmol/L min^{-1}), corresponding to the main circulating phenolic metabolites detected in the plasma. A total of 25 phenolic metabolites were identified in the plasma from 0 to 24 h. The time-course concentrations of all the phenolic metabolites in the plasma are provided in **Supplementary Table 2S**. Dihydroxyphenylacetic acid sulphate was the most abundant metabolite, followed by FA sulphate, (methyl) catechol sulphate, dihydroxyphenylacetic acid, and hydroxybenzoic acid.

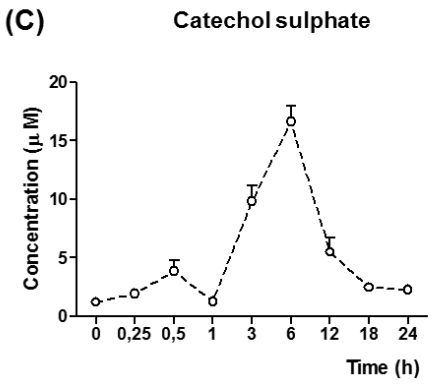
The circulating phenolic metabolites showed two different kinetics of absorption according to their time of appearance in the plasma (see t_{max} in **Figure 1A**). The two groups showed biphasic behaviour (from 0 to 24 h) with two maximum absorption peaks but different t_{max} (**Figures 1B and 1C**). In the first group, represented by dihydroxyphenylacetic acid sulphate, the maximum absorption occurs 30 min after ingestion of the RBEE extract. Then, their concentrations decreased until 6 h, and a second absorption peak is observed at a lower concentration at 12 h (**Figure 1B**). The second group of phenolic compounds detected in the plasma, represented by catechol sulphate, showed the first absorption peak 30 min after ingestion of the RBEE extract and the second peak at 6 h (**Figure 1C**), reaching a higher plasma concentration compared with the first group of compounds.

(A)

Phenolic compound	AUC (µmol/L min^{-1})	C_{max} (µM)	t_{max} (min)
Ferulic acid	336.65 ± 25.80	1.63 ± 0.33	27.00 ± 3.00
Isoferulic acid	17.51 ± 6.51	0.28 ± 0.19	24.00 ± 3.68
Ferulic acid sulphate	3748.03 ± 150.53	13.86 ± 3.46	33.00 ± 7.35
Ferulic acid glucuronide	210.75 ± 8.57	0.74 ± 0.21	36.00 ± 6.00
p-coumaric acid	395.90 ± 47.73	2.32 ± 0.65	36.00 ± 6.00
Coumaric acid sulphate	946.78 ± 25.66	3.91 ± 0.78	24.00 ± 3.68
Coumaric acid glucuronide	6.80 ± 0.93	0.05 ± 0.02	30.00 ± 0.00
Hydroxyphenylacetic acid	1092.48 ± 64.59	2.10 ± 0.32	21.00 ± 9.93
Hydroxyphenylacetic acid glucuronide	81.49 ± 25.05	1.90 ± 0.43	24.00 ± 3.68
Dihydroxyphenylacetic acid	6186.37 ± 471.22	13.17 ± 2.54	24.00 ± 3.68
Dihydroxyphenylacetic acid sulphate	45657.95 ± 1894.32	160.27 ± 27.16	24.00 ± 3.68
Dihydroxyphenylacetic acid glucuronide	761.14 ± 121.48	2.60 ± 0.70	21.00 ± 3.68
Dihydroxyphenylpropionic acid glucuronide	57.34 ± 5.39	0.11 ± 0.01	33.00 ± 7.35
p-hydroxybenzoic acid	135.90 ± 15.25	0.80 ± 0.27	27.00 ± 3.00
Hydroxybenzoic acid sulphate	277.60 ± 14.38	0.48 ± 0.07	33.00 ± 7.35
Dihydroferulic acid	56.69 ± 19.30	0.20 ± 0.07	291.00 ± 69.00
Dihydroferulic acid sulphate	596.18 ± 93.62	1.42 ± 0.34	228.00 ± 80.83
Phenylacetic acid	380.45 ± 76.88	1.08 ± 0.22	372.00 ± 191.77
Hydroxyphenylpropionic acid	209.08 ± 27.32	0.80 ± 0.14	228.00 ± 80.83
Hydroxyphenylpropionic acid sulphate	3132.60 ± 177.53	5.22 ± 0.78	294.00 ± 66.00
Catechol sulphate	9473.87 ± 701.10	16.65 ± 1.30	360.00 ± 0.00
Methyl catechol sulphate	8015.91 ± 704.37	13.80 ± 1.40	360.00 ± 0.00
Methyl catechol glucuronide	213.87 ± 48.56	0.45 ± 0.06	294.00 ± 66.00
Hydroxybenzoic acid	6344.40 ± 445.91	13.38 ± 1.31	360.00 ± 0.00
Hippuric acid	764.61 ± 34.25	0.93 ± 0.05	297.00 ± 129.94



Others: Ferulic acid
Isoferulic acid
Ferulic acid sulphate
Ferulic acid glucuronide
p-coumaric acid
Coumaric acid sulphate
Coumaric acid glucuronide
Hydroxyphenylacetic acid
Hydroxyphenylacetic acid glucuronide
Dihydroxyphenylacetic acid
Dihydroxyphenylacetic acid glucuronide
Dihydroxyphenylpropionic acid glucuronide
p-hydroxybenzoic acid
Hydroxybenzoic acid sulphate



Others: Dihydroferulic acid
Dihydroferulic acid sulphate
Hydroxyphenylpropionic acid
Hydroxyphenylpropionic acid sulphate
Phenylacetic acid
Hydroxybenzoic acid
Methyl catechol sulphate
Methyl catechol glucuronide
Hippuric acid

Figure 1. Plasma phenolic kinetic parameters expressed as the area under the curve (AUC), maximum peak concentration (C_{max}) and the time at which the C_{max} is observed (t_{max}) **(A).** Mean concentration (µM) *versus* time of the main phenolic metabolites detected in rat plasma samples after the acute intake of RBEE generated with an early **(B)** or late **(C)** t_{max}.

3.2 Determination of ferulic acid-derived phenolic metabolites in urine

The main circulating phenolic metabolites identified in the plasma samples were also found in the urine samples from 0 to 48 h. **Figure 2** shows the main phenolic compounds excreted in the urine (µmol) at different times after ingestion of the RBEE extract: from 0-4 h, 4-9 h, 9-24 h, and 24-48 h. The main phenolic metabolites excreted were FA sulphate, hydroxyphenylpropionic acid and its sulphate form, dihydroxyphenylacetic acid sulphate, and (methyl) catechol sulphate. As can be seen, the highest urine excretion was observed between 9 to 24 h and between 24 to 48 h, in comparison with 0-9 h after the RBEE intake.

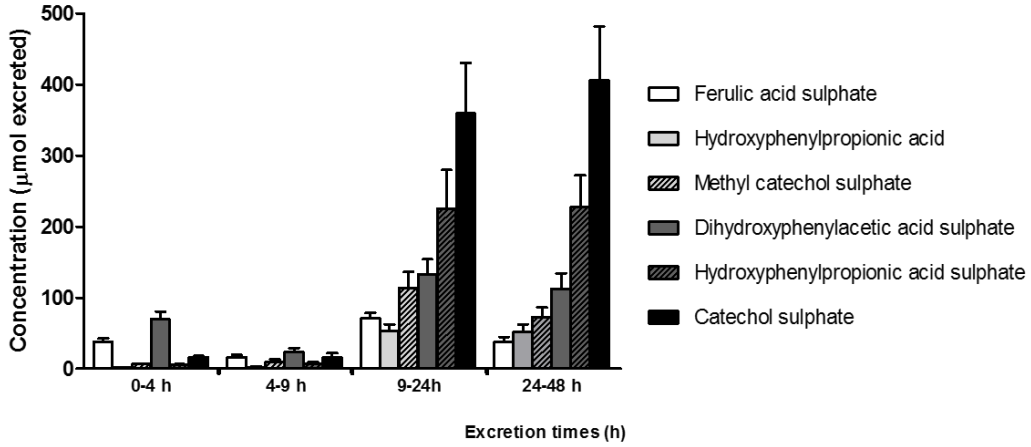


Figure 2. Urine molar excretion (µmols) of the main phenolic metabolites observed after the acute intake of RBBE.

3.3 Determination of ferulic acid-derived phenolic metabolites in faeces

Regarding the analysis of the faeces, different catabolic metabolites were determined from 0 to 48 h after the RBEE intake **(Table 1)**. All the phenolic metabolites detected, with the exception of dihydroxyphenylacetic acid sulphate, were unconjugated simple aromatic acids. Hydroxyphenylpropionic acid, followed by dihydroxyphenylacetic acid and dihydroferulic acid, were the main catabolic metabolites (end products) quantified in the faeces **(Table 1)**.

3.4 RBEE phenolic metabolites attenuate superoxide production

Superoxide production induced by ET-1 was measured by lucigenin-enhanced luminescence in aortic rings. The tissue was incubated with the plasma of rats sacrificed after

Table 1. Faecal phenolic metabolites. Concentration of phenolic metabolites detected in rat faeces (µmol/g) after acute intake of rice bran enzymatic extract.

Phenolic metabolite (µmol/ g faeces)	**0 h**	**24 h**	**36 h**	**48 h**
Ferulic acid	3.37 ± 1.39	3.75 ± 1.60	5.08 ± 2.05	5.60 ± 1.76
Dihydroferulic acid	3.86 ± 1.96	3.88 ± 1.61	5.71 ± 2.20	8.05 ± 2.42
Ferulic acid sulphate	0.01 ± 0.02	0.03 ± 0.07	0.31 ± 0.84	0.01 ± 0.02
Caffeic acid	0.05 ± 0.03	0.09 ± 0.03	0.09 ± 0.03	0.09 ± 0.02
p-coumaric acid	0.18 ± 0.04	0.18 ± 0.04	0.25 ± 0.08	0.29 ± 0.05
Vanillic acid	n.d.	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.03
Dihydroxyphenylacetic acid	6.80 ± 1.13	11.67 ± 2.75	8.93 ± 2.35	7.60 ± 2.61
Dihydroxyphenylacetic acid sulphate	n.d.	0.20 ± 0.55	3.61 ± 10.20	0.10 ± 0.30
Hydroxyphenylpropionic acid	24.67 ± 13.14	77.45 ± 19.70	56.25 ± 13.95	66.85 ± 23.42
Dihydroxyphenylpropionic acid (dihydrocaffeic acid)	n.d.	0.02 ± 0.03	n.d.	0.01 ± 0.02
Protocatechuic acid	n.d.	0.72 ± 0.47	0.48 ± 0.52	0.46 ± 0.38
Hydroxybenzoic acid	0.90 ± 0.30	3.09 ± 1.05	2.81 ± 1.36	3.18 ± 0.58
Hydroxybenzoic acid sulphate	0.12 ± 0.35	0.10 ± 0.28	0.78 ± 1.93	n.d.

30 min of RBEE gavage, or in HEPES-KHS buffer in the presence of RBEE, isolated FA and ORZ, respectively (**Figure 3**). Superoxide production significantly increased after ET-1 incubation (p<0.001) in both the plasma- and HEPES-KHS-incubated rings. The phenolic compound-enriched plasma (30 min after RBEE intake) completely inhibited the ET-1-induced NADPHox activation (p<0.001) (**Figure 3A**). Moreover, the incubation with RBEE and FA also reduced ET-1-induced superoxide production (p<0.05), in contrast to ORZ (**Figure 3B**).

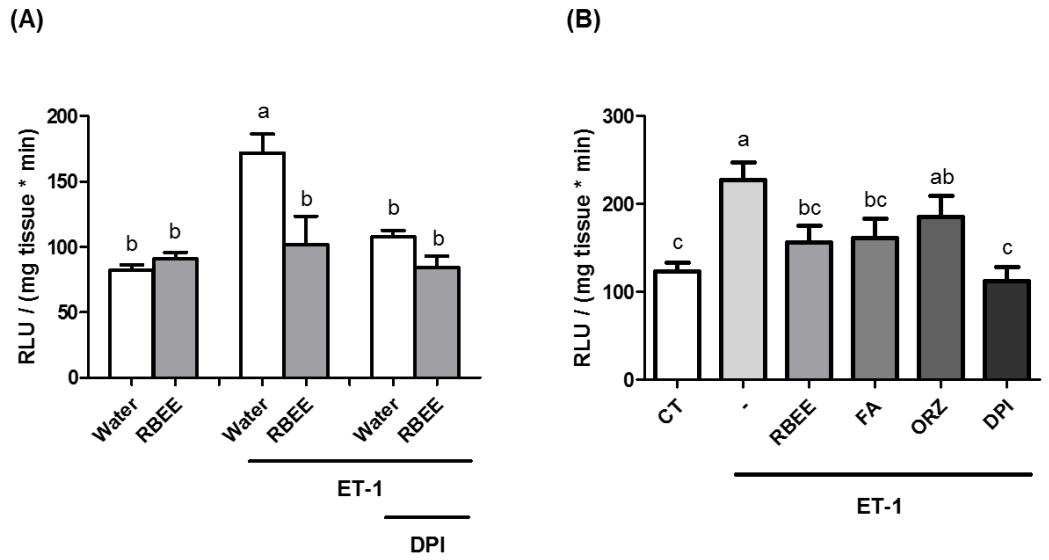


Figure 3. Superoxide production is reduced by RBEE phenolic metabolites. Superoxide production of aortic rings incubated in rat plasma after oral gavage (30 min) of rice bran enzymatic extract (RBEE) or water (CT) in the presence or absence of endothelin-1 (ET-1, 10 nmol/L) or diphenylleneiodininum (DPI, 10 µmol/L) **(A)**. Superoxide production of ET-1 exposed aortic rings incubated in HEPES-KHS buffer in the presence or absence of RBEE (367.6 mg/L), FA (5.5 µmol/L), ORZ (5.5 µmol/L) or DPI **(B)**. Values were mean±SEM (n=5). Columns with different letters differ significantly (one-way ANOVA followed by Bonferroni).

4. Discussion

FA is a ubiquitous phenolic molecule found in the cell walls of the seeds and leaves of monocotyledon plants both in its free form and conjugated to polysaccharides, glycoproteins, polyamines and fatty acids. Rice bran is one of the main sources of ferulic acid, found as a part of the γ-oryzanol molecule. Both molecules are known for their antioxidant activities. However, their bioavailability and metabolic pathways remain unknown. Therefore, we aimed to evaluate the bioavailability and the metabolic routes of the ferulic acid-derived compounds after oral consumption of a rice bran enzymatic extract (RBEE) and to study their antioxidant activity on the NADPHox complex at the concentrations found in the plasma.

After RBEE consumption, a good number of phenolic compounds derived from FA were identified in plasma, urine and faeces. Free FA (351± 5mg/kg RBEE) is present in the RBEE used in the present study, the ORZ (FA conjugated form) being an additional source (8950 ± 850 mg/kg RBEE). The study of ORZ absorption has been gathering interest since the early 1980s. Fujiwara et al. reported the metabolism and oral absorption of ORZ, choosing a ^{14}C-labelled located in the FA to motorize ORZ [22, 23]. However, the study of the bioavailability of the whole ORZ molecule has been addressed without success to date [24]. In contrast, ferulic acid moiety has been identified in plasma after ORZ intake [23, 25]. The low or null ORZ absorption could be related to the high molecular weight, or to its low solubility in water, but more importantly, it could be the consequence of the esterase activity in the small and large intestine leading to OZ cleavage into ferulic acid which can be now absorbed [26, 27].

The phenolic compounds detected in rat plasma in the present study followed biphasic behaviour with two different profiles, following an early or late C_{max} peak (**Figure 1**). The group with an early t_{max} followed by a second peak of lower magnitude could be explained by gastro-intestinal absorption followed by the re-absorption through the enterohepatic recycling circulation. On the other hand, the late t_{max} group could be assigned to molecules that suffer higher colonic microbial metabolism, which would also explain the lower plasma concentration, in comparison with the first group. The results obtained were in agreement with the studies reported in the literature for the analysis of the bioavailability of hydroxycinnamic acids after the consumption of coffee or herbs (such as thyme, which is rich in rosmarinic acid) [28-30]. In these studies, the metabolites identified could also be classified into two groups: in one group, the metabolites absorbed in the small intestine, which were mainly phase-II metabolites (sulphated and glucuronidated), such as caffeic and ferulic acid conjugates; and in the second group, the phenolic compounds absorbed

in the large intestine (colonic origin), such as dihydrocaffeic and dihydroferulic acids. As in our study, coumaric acid sulphate and ferulic acid in its sulphate and glucuronide forms were also reported in the plasma samples (0 to 6 h) after the acute intake of a thyme phenolic extract [31], while the generation of dihydro ferulic acid from FA has been reported to occur in the colon as a consequence of the microflora of the large intestine by mutant strains of *Pseudomonas fluorescens* [32].

The study of FA-derived phenolic metabolites in urine and faeces was aimed at identifying the RBEE metabolic pathways. Most of the metabolites found in plasma were also identified in urine, indicating a fast metabolization rate. As in our study, hydroxyphenylpropionic acid sulphate was identified in urine samples after a sustained daily intake for 3 weeks of olive oil enriched with its phenolic compounds plus complementary phenolics from thyme, and it was selected as one of the best compliance biomarkers for thyme phenolic intake [33]. Similarly, FA sulphate was the main metabolite excreted in urine after oral administration of γ-oryzanol-^{14}C in rats [22].

Regarding faeces, all the metabolites detected were simple phenolic acids with the exception of dihydroxyphenylacetic acid sulphate. These phenolic compounds could be formed from phase-II metabolites secreted via the biliary route into the duodenum, where they are subjected to the action of bacterial enzymes, especially β-glucuronidase, in the distal segments of the intestine, after which they may be reabsorbed. This enterohepatic recycling may lead to a longer presence of phenolic metabolites in the body, explaining the maximum plasma concentration observed at 6 or 12 h after the RBEE intake (**Figure 1C**). Similarly, hydroxyphenylpropionic and dihydroferulic acids were also reported to be the main metabolites formed after the *in-vitro* colonic fermentation of coffee with human faecal inocula [34]. Additionally, hydroxyphenylpropionic acid was also reported to be the main catabolic metabolite when different sources of caffeic acid, such as rosmarinic acid [35], caffeic acid [36], chlorogenic acid [36, 37] and caftaric acid [36], were incubated with human faecal samples *in vitro*. Other study by Pereira-Caro et al. showed that after the *in-vitro* colonic fermentation of FA, methoxy hydroxyphenylpropionic and hydroxyphenylpropionic acids were the main catabolites formed [38]. Nevertheless, the methoxy form of hydroxyphenylpropionic acid was not detected in faeces in our study.

In order to understand the generation of the phenolic metabolites identified in the different biological samples (plasma, urine and faeces) in the present study, different metabolic pathways have been proposed (**Figure 4**). After the RBEE intake, the phenolic metabolites generated were mainly sulphated, glucuronided and methylated conjugates formed through the action of sulphotransferases (SULFs), uridine-5'-diphosphate

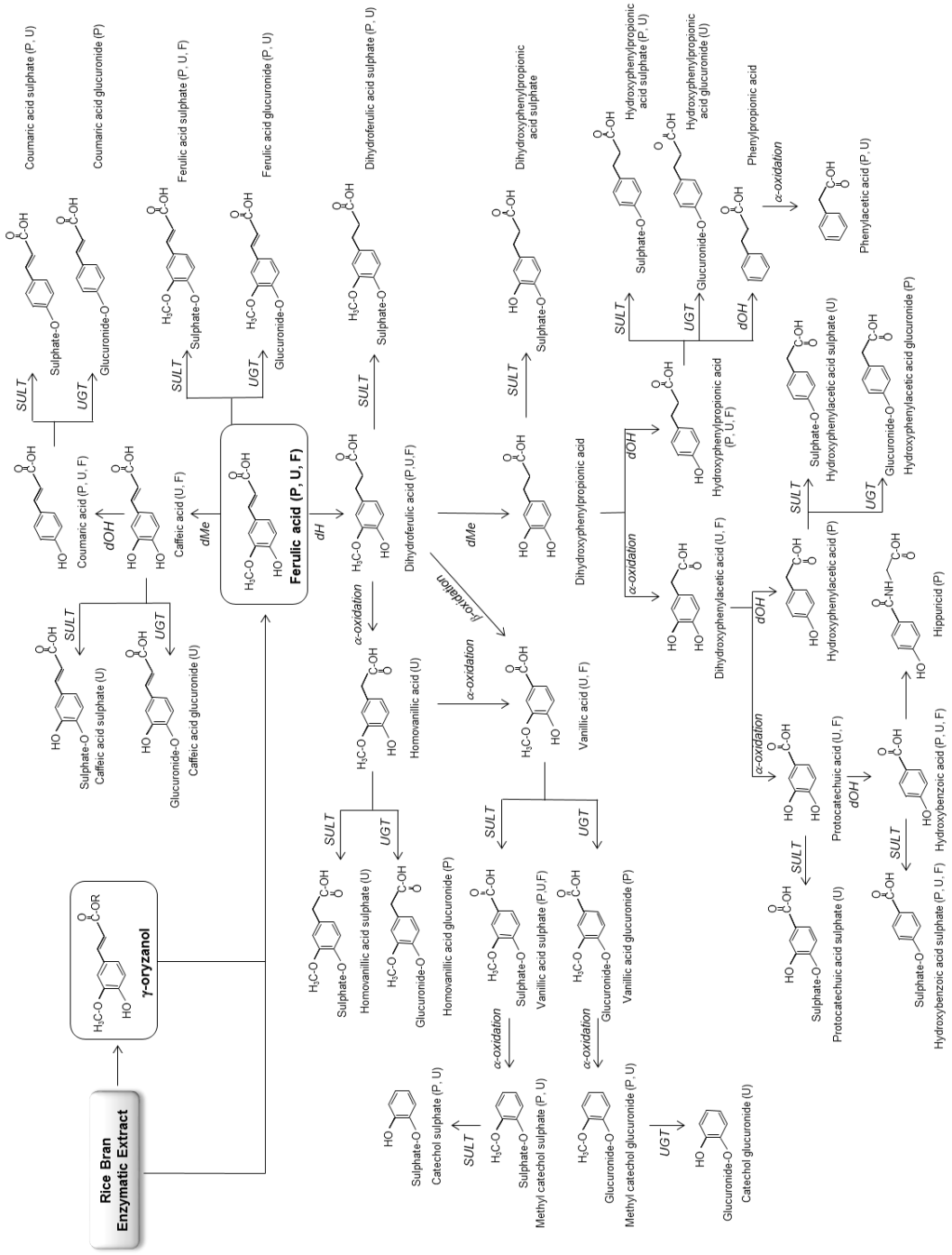


Figure 4. Metabolic pathways proposed for the phenolic metabolites generated from ferulic acid from rice bran enzymatic extract. *dH*, dehydrogenation (reductase); *dOH*, dehydroxylation; *dMe*, demethylation; a-*oxidation*, one decarboxylation; *SULT*, sulphotransferase; *UGT*, glucuronosyl-transferase; and *COMT*, catechol-O-methyltransferase.

glucuronosyl-transferases (UGT), and catechol-O-methyltransferases (COMT), respectively. Nevertheless, sulphation was the main phase-II metabolic transformation. On the other hand, part of the phenolic metabolites were not absorbed in the upper gastrointestinal tract and reached the colon, where they could be extensively metabolized by microflora enzymes resulting in a further modification of their chemical structure. Decarboxylation (a-oxidation) and dehydroxylation were the main reactions that may be carried out. The microbial catabolites generated could then undergo phase-II metabolism locally and/or be absorbed and reach the liver, where they would be subject to enzymatic metabolism before re-entering the systemic blood circulation and finally being excreted in urine.

Related to the superoxide production, FA- and ORZ-rich products have previously been reported to exert exceptional antioxidant activities *in vitro* [40] and *in vivo* [41]. Here we reported the biological value of the ferulic acid-derived metabolites formed after the RBEE intake on NADPHox inhibition. *In-vitro* incubation of aortic rings with RBEE and FA at the concentrations that correspond to the AUC of a 24-h period (**Figure 3B**) completely inhibited ET-1-induced superoxide production. Moreover, incubation with plasma enriched with the naturally occurring ferulic acid metabolites obtained after 30 min (**Supplementary Table 1S**) RBEE gavage gave the same result (**Figure 3A**). However, the FA concentration at the t_{max} (30 min) was lower than the AUC concentration (**Figure 1A**), which indicates that some of the FA metabolites present in plasma at 30 min (t_{max}) are active as well. FA has shown downregulation of NADPHox subunits in rat VSMCs exposed to H_2O_2 through reduction of NF-κβ activation [42], while RBEE diet supplementation reduced the expression of NADPHox subunits in the aorta and small mesenteric arteries of ApoE-/- mice [13, 14] and Zucker rats [16, 17], confirming the biological activity of RBEE as source of FA. However, the scavenging potential of FA and its metabolites and a direct molecular interaction should not be dismissed since the incubation period in the present study was short enough to achieve expression modifications of the NADPHox subunits [43].

5. Conclusions

In conclusion, in this work, the oral bioavailability and metabolic pathways of FA-derived metabolites from a rice bran enzymatic extract (RBEE) is described for the first time. Based on the analysis of plasma after the RBEE intake, the metabolism of FA was characterized by its fast biphasic absorption and metabolization. Two groups of FA metabolites were identified according to the plasma pharmacokinetic parameters (C_{max} and t_{max}), which were governed by enterohepatic recirculation and colonic bacterial metabolization processes. The reduction of the superoxide production reinforces the interest in RBEE as a nutraceutical ingredient in the prevention of oxidative stress-related processes.

Conflict of interest

All authors declare no conflict of interest concerning the content of the manuscript.

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Supplementary Table 1S. SRM conditions used for the quantification of phenolic metabolites by UPLC-MS/MS.

Phenolic compound	MW	Quantification			Standard used for quantification
		SRM	Cone voltage (V)	Collision energy (eV)	
Catechol	110	108.9 > 90.9	40	15	Catechol
Methyl catechol	124	123 > 109	40	15	Catechol
Catechol sulphate	190	189 > 109	20	15	Catechol
Catechol glucuronide	286	285 > 123	40	15	Catechol
Methyl catechol sulphate	204	203 > 123	20	15	Catechol
Methyl catechol glucuronide	300	299 > 123	40	15	Catechol
Hydroxybenzoic acid	138	137 > 93	30	15	4-Hydroxybenzoic acid
Hydroxybenzoic acid sulphate	218	317 > 137	35	15	4-Hydroxybenzoic acid
Hydroxybenzoic acid glucuronide	314	313 > 137	35	15	4-Hydroxybenzoic acid
Protocatechuic acid	154	153 > 109	40	15	Protocatechuic acid
Protocatechuic acid sulphate	234	233 > 153	40	15	Protocatechuic acid
Protocatechuic acid glucuronide	330	329 > 153	40	20	Protocatechuic acid
Hydroxytyrosol	154	153 > 123	35	15	Protocatechuic acid
Hydroxytyrosol sulphate	234	233 > 153	35	15	Protocatechuic acid
Hydroxytyrosol glucuronide	330	329 > 153	35	15	Protocatechuic acid
Phenylacetic acid	136	135 > 91	20	5	Phenylacetic acid
p-Hydroxyphenylacetic acid	152	151 > 107	20	10	4-Hydroxyphenylacetic acid
m-Hydroxyphenylacetic acid	152	151 > 107	20	10	4-Hydroxyphenylacetic acid
o-Hydroxyphenylacetic acid	152	151 > 107	20	10	4-Hydroxyphenylacetic acid
Hydroxyphenylacetic acid sulphate	232	231 > 151	40	15	4-Hydroxyphenylacetic acid
Hydroxyphenylacetic acid glucuronide	328	327 > 151	40	15	4-Hydroxyphenylacetic acid
Dihydroxyphenylacetic acid	168	167 > 123	20	10	3,4-Dihydroxyphenylacetic acid
Dihydroxyphenylacetic acid sulphate	248	247 > 167	35	15	3,4-Dihydroxyphenylacetic acid
Dihydroxyphenylacetic acid glucuronide	344	343 > 167	40	25	3,4-Dihydroxyphenylacetic acid
Phenylpropionic acid	150	149 > 105	20	5	3-(4-Hydroxyphenyl)propionic acid
Hydroxyphenylpropionic acid	166	165 > 121	20	10	3-(4-Hydroxyphenyl)propionic acid
Hydroxyphenylpropionic acid sulphate	246	245 > 165	35	15	3-(4-Hydroxyphenyl)propionic acid
Hydroxyphenylpropionic acid glucuronide	342	341 > 165	40	25	3-(4-Hydroxyphenyl)propionic acid
Dihydroxyphenylpropionic acid (dihydrocaffeic acid)	181	181 > 137	20	15	3-(2,4-Dihydroxyphenyl)propionic acid
Dihydroxyphenylpropionic acid sulphate	262	261 > 181	40	15	3-(2,4-Dihydroxyphenyl)propionic acid
Dihydroxyphenylpropionic acid glucuronide	358	357 > 181	40	10	3-(2,4-Dihydroxyphenyl)propionic acid
Vanillic acid	168	167 > 123	30	10	Vanillic acid

Vanillic acid sulphate	248	247 > 167	40	10	Vanillic acid
Vanillic acid glucuronide	344	343 > 167	40	10	Vanillic acid
Homovanillic acid	182	181 > 137	25	10	Vanillic acid
Homovanillic acid sulphate	262	261 > 181	40	15	Vanillic acid
Homovanillic acid glucuronide	358	357 > 181	40	20	Vanillic acid
Caffeic acid	180	179 > 135	35	10	Caffeic acid
Caffeic acid sulphate	260	259 > 179	35	15	Caffeic acid
Caffeic acid glucuronide	356	355 > 179	40	15	Caffeic acid
Coumaric acid	164	163 > 119	35	10	*p*-coumaric acid
Coumaric acid sulphate	244	243 > 163	35	15	*p*-coumaric acid
Coumaric acid glucuronide	340	339 > 163	35	15	*p*-coumaric acid
Ferulic acid	194	193 > 134	30	15	Ferulic acid
Ferulic acid sulphate	274	273 > 193	35	15	Ferulic acid
Ferulic acid glucuronide	370	369 > 193	35	15	Ferulic acid
Dihydroferulic acid	196	195 > 136	35	10	Ferulic acid
Dihydroferulic acid sulphate	276	275 > 195	35	15	Ferulic acid
Dihydroferulic acid glucuronide	372	371 > 195	35	20	Ferulic acid
Hippuric acid	179	178 > 134	40	10	Caffeic acid
Hydroxyhippuric acid	195	194 > 100	40	10	Caffeic acid

MW: molecular weight (g/mol)

Supplementary Table 2S. Time-course concentrations of the phenolic metabolites found in plasma after oral gavage of rice bran enzymatic extract.

Phenolic metabolites (μmol/L plasma)	Time after RBEE oral administration							
	0 h	15 min	30 min	1 h	3 h	6 h	12 h	18 h	24 h
Ferulic acid	n.d.	1.26±0.28	1.58±0.35	0.57±0.08	0.38±0.07	0.08±0.01	0.45±0.05	0.00±0.0	n.d.
Isoferulic acid	n.d.	0.05±0.03	0.25±0.19	0.01±0.00	0.00±0.00	0.00±0.00	0.03±0.01	n.d.	n.d.
Dihydroferulic acid	n.d.	0.03±0.02	0.05±0.02	0.01±0.00	0.00±0.00	0.19±0.07	0.01±0.01	n.d.	n.d.
Ferulic acid sulphate	n.d.	9.61±1.11	12.49±3.95	6.00±0.70	5.57±0.58	1.27±0.13	4.67±0.18	0.04±0.02	n.d.
Ferulic acid glucuronide	n.d.	0.37±0.06	0.73±0.23	0.29±0.03	0.29±0.03	0.07±0.01	0.29±0.03	n.d.	n.d.
Dihydroferulic acid sulphate	n.d.	0.69±0.13	0.84±0.29	0.33±0.06	0.48±0.16	1.32±0.36	0.30±0.03	0.02±0.01	n.d.
p-coumaric acid	n.d.	1.56±0.48	2.25±0.68	0.96±0.12	0.45±0.04	0.04±0.01	0.48±0.08	0.00±0.00	n.d.
Coumaric acid sulphate	0.02±0.01	2.92±0.41	3.54±0.92	1.40±0.17	1.24±0.11	0.28±0.02	1.24±0.06	0.02±0.00	0.01±0.00
Coumaric acid glucuronide	n.d.	0.02±0.00	0.05±0.01	0.01±0.00	0.01±0.00	0.00±0.00	0.01±0.00	n.d.	n.d.
Hydroxyphenylacetic acid	0.75±0.24	1.81±0.47	1.70±0.35	0.66±0.05	0.78±0.13	1.32±0.17	0.67±0.09	0.46±0.04	0.50±0.08
Hydroxyphenylacetic acid glucuronide	n.d.	0.99±0.28	1.39±0.62	0.47±0.22	0.00±0.00	n.d.	n.d.	n.d.	n.d.
Dihydroxyphenylacetic acid sulphate	0.62±0.38	111.8±16.3	125.7±38.1	92.45±16.2	59.27±5.57	19.03±2.70	53.65±2.58	1.71±0.28	1.44±0.21
Dihydroxyphenylacetic acid glucuronide	n.d.	1.66±0.18	2.51±0.73	1.20±0.16	0.82±0.22	0.64±0.17	0.82±0.21	n.d.	n.d.
Hydroxyphenylpropionic acid sulphate	0.03±0.02	1.76±0.45	2.85±0.89	1.80±0.51	3.60±0.55	5.22±0.78	2.64±0.26	0.00±0.00	n.d.
Dihydroxyphenylpropionic acid glucuronide	0.01±0.01	0.08±0.00	0.10±0.01	0.07±0.00	0.06±0.00	0.06±0.02	0.07±0.00	n.d.	n.d.
p-hydroxybenzoic acid	n.d.	0.33±0.09	0.58±0.17	0.23±0.03	0.10±0.01	0.11±0.03	0.16±0.04	n.d.	n.d.
Hydroxybenzoic acid	n.d.	3.33±0.85	5.04±1.09	3.42±0.29	4.45±0.45	13.37±1.31	4.35±0.55	0.22±0.16	n.d.
Hydroxybenzoic acid sulphate	n.d.	0.34±0.04	0.43±0.10	0.29±0.04	0.26±0.01	0.34±0.01	0.28±0.04	0.02±0.00	0.01±0.00
Catechol sulphate	1.22±0.09	1.95±0.31	3.87±0.94	1.25±0.25	9.82±1.33	16.65±1.30	5.50±1.23	2.50±0.16	2.25±0.25
Methyl catechol sulphate	0.17±0.11	3.51±0.75	6.07±2.29	2.88±0.63	23.23±16.01	13.80±1.40	5.88±0.88	1.03±0.12	0.83±0.14
Hippuric acid	0.30±0.02	0.76±0.07	0.75±0.10	0.36±0.03	0.62±0.04	0.90±0.09	0.64±0.09	0.25±0.01	0.26±0.02

n.d.: not detected

CAPÍTULO VII

Quien enseña al hombre a morir, le enseña a vivir

(Michel de Montaigne, 1533-1592)

EL ÁCIDO FERÚLICO, COMPONENTE BIOACTIVO DEL SALVADO DE ARROZ, MEJORA EL ESTRÉS OXIDATIVO Y EL CICLO MITOCONDRIAL EN RATONES Y EN CÉLULAS MONONUCLEARES HUMANAS

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En revision en *Journal of Nutritional Biochemistry*

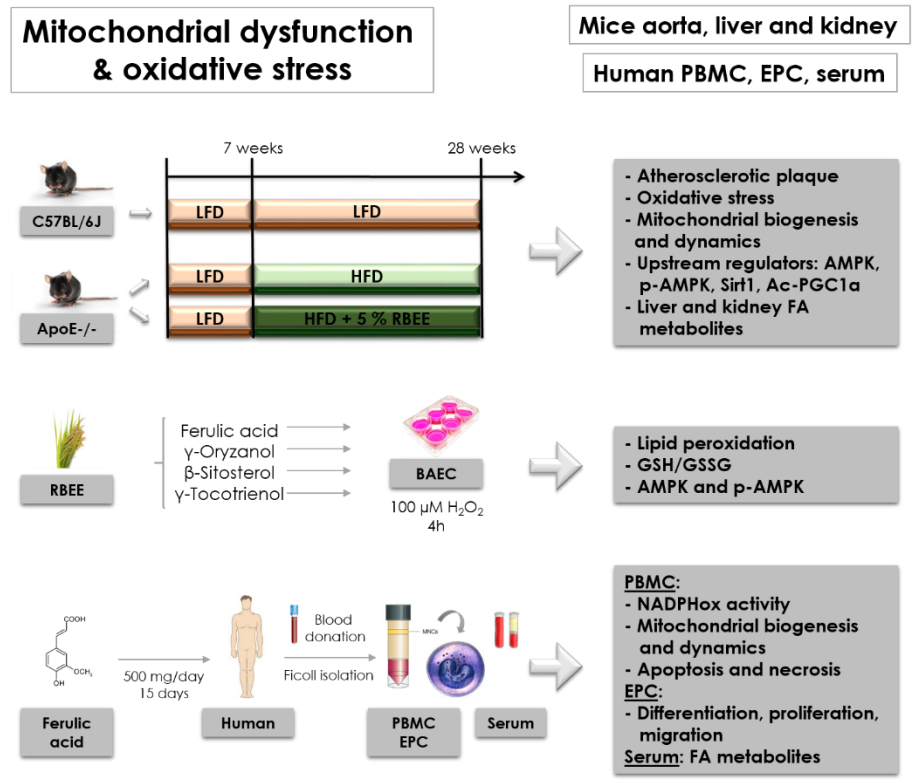
El ácido ferúlico ha mostrado una potente capacidad antioxidante, capaz de reducir el desarrollo de aterosclerosis en modelos animales. Además, se conoce que existe una relación recíproca entre el desarrollo de aterosclerosis, la disfunción mitocondrial y el estrés oxidativo. Por ello, el objetivo de este trabajo fue la caracterización de los efectos a nivel molecular de los efectos un extracto enzimático de salvado de arroz (EESA) y de sus principales componentes bioactivos en el estrés oxidativo y disfunción mitocondrial asociada al proceso aterosclerótico en ratones ApoE-/- y de uno de los componentes del EESA, el ácido ferúlico, en humanos.

Ratones ApoE-/- de 7 semanas de edad fueron alimentados durante 21 semanas con una dieta alta en grasa y colesterol (HFD), suplementada o no al 5% con EESA. Como grupo control se emplearon ratones de genotipo salvaje (C57BL/6J) alimentados con dieta baja en grasa y colesterol (LFD).

La suplementación de la dieta con el EESA redujo el desarrollo de la placa aterosclerótica y el estrés oxidativo en la aorta, como muestra el menor ratio de glutatión/glutatión oxidado (GSH/GSSG), el menor estado de oxidación de peroxiredoxina ($Prx\text{-}SO_3$) y de peroxidación lipídica. Además, el EESA previno la reducción del ARN mensajero, inducida por la dieta HFD, en la expresión de marcadores de la biogénesis (*Pgc-1α, Pgc-1β, Nrf-1*) y dinámica (*Mfn1, Mnf2, Fis1, Beclin-1*) del ciclo mitocondrial. La suplementación dietética con EESA también incrementó la fosforilación de AMPK, induciendo una mayor deacetilación de PGC-1α, aumentando así la biogénesis mitocondrial. En el análisis *in vitro* en cultivos de células endoteliales de aorta bovina (BAEC) con los principales compuestos bioactivos presentes en el EESA, se identificó al ácido ferúlico como principal responsable de los efectos observados. El ácido ferúlico y sus metabolitos fueron identificados en hígado y riñones, confirmando su absorción desde el tracto intestinal. Con objeto de confirmar estos resultados en humanos, se estudió la biodisponibilidad y metabolismo en plasma del ácido ferúlico tras la administración de 500

mg de ácido ferúlico de forma aguda y los efectos en células polimorfonucleares (PBMC) y células progenitoras endoteliales (EPC) tras el consumo de 500 mg de ácido ferúlico por día, durante 15 días. El consumo de ácido ferúlico incrementó la expresión de ARN mensajero de PGC-1α y MNF1 y redujo la actividad de NADPH oxidasa, la liberación de anión superóxido, y la apoptosis y necrosis de PBMC. Además, se mejoró la diferenciación y proliferación de EPC.

Con todo esto, podemos concluir que el ácido ferúlico, identificado como uno de los mayores componentes bioactivos del EESA, es capaz de mejorar la biogénesis y dinámica del ciclo mitocondrial y de reducir el estrés oxidativo en la aorta de ratones ApoE-/- y de PBMC humanas.



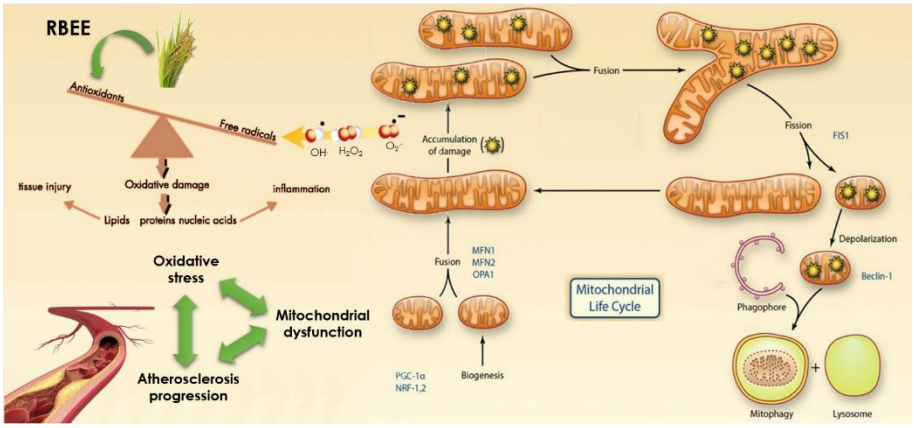


Figura 21: Diseño experimental y resumen de resultados.

FERULIC ACID, A BIOACTIVE COMPONENT OF RICE BRAN, IMPROVES OXIDATIVE STRESS AND MITOCHONDRIAL BIOGENESIS AND DYNAMICS IN MICE AND IN HUMAN MONONUCLEAR CELLS

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Abstract

Objective: To characterize the molecular effects of rice bran and its components on vascular oxidative stress and mitochondrial dysfunction during atherogenesis in mice and in humans.

Methods and results: ApoE-/- mice were fed a high-fat, high-cholesterol diet (HFD) or HFD supplemented with 5% rice bran enzymatic extract (RBEE) for 21 weeks. RBEE prevented development of atherosclerotic plaques and oxidative stress in mouse aorta, as exemplified by increased GSH/GSSG ratio, reduced peroxiredoxin-SO_3 and reduced lipid peroxidation. RBEE prevented the HFD-mediated downregulation of mRNA expression of markers of mitochondrial biogenesis (*Pgc-1α, Pgc-1β, Nrf-1*) and dynamics (*Mfn1, Mfn2, Fis1, Beclin-1*). Moreover, RBEE increased p-AMPK leading to increased deacetylation of PGC-1α. Analysis of the bioactive components of RBEE in bovine aortic endothelial cells identified ferulic acid as the component responsible for the observed effects. Ferulic acid metabolites such as sulfonates and glucuronides significantly increased in murine liver and kidney after treatment. To confirm these findings in humans, the kinetics of ferulic acid and its metabolites were determined in healthy volunteers who consumed 500 mg ferulic acid/day for 15 days. Ferulic acid intake reduced NADPH oxidase activity, superoxide release, apoptosis and necrosis and increased PGC-1α and MFN1 mRNA expression in human peripheral blood mononuclear cells. Moreover, differentiation and proliferation of endothelial progenitor cells were improved.

Conclusion: Ferulic acid was identified as a major active component of rice bran, which improved mitochondrial biogenesis and dynamics and reduced oxidative stress in mouse aorta and human mononuclear cells.

Keywords: Rice bran enzymatic extract; ferulic acid; atherosclerosis; mitochondrial function, oxidative stress.

1. Introduction

Reactive oxygen species (ROS) contribute to atherogenesis by inducing oxidative modifications of lipoproteins, endothelial dysfunction, initiation of vascular inflammation, thrombosis and smooth muscle cell proliferation [1]. Since mitochondria are both sources and targets of ROS, uncoupling of mitochondrial oxidative phosphorylation can result in the generation of superoxide production leading to oxidative modifications of mitochondrial proteins and lipids and to mutations in the mitochondrial DNA [2-4].

Mitochondrial structure and function are preserved by mitochondrial biogenesis and dynamics [5]. Peroxisome proliferative activated receptor-α coactivator 1 (PGC-1α) is a master regulator of mitochondrial biogenesis [6, 7]. PGC-1α induces expression of its downstream factors NRF-1, -2 and TFAM. NRF-1 regulates the expression of nuclear genes that control the respiratory chain as well as TFAM, which in turn regulates the mitochondrion-encoded cytochrome c oxidase subunit genes [7]. PGC-1α activity is controlled though post-transcriptional modifications by metabolic sensors such as sirtuin 1 (SIRT1) or AMP-activated protein kinase (AMPK) to adapt mitochondrial biogenesis to energy expenditure [8, 9]. Mitochondrial morphology is determined by the dynamic processes of fusion and fission. Both functions are tightly regulated to prevent excessive fragmentation or elongation of mitochondria. Fusion is mediated by mitofusin (MFN) 1, MFN2, and optic atrophy protein 1 (OPA1), and fission is regulated by dynamin-related protein-1 (DRP1) and fission 1 (FIS1) [10].

Mitochondria and oxidative stress play a key role for the balance of cellular survival, regeneration and apoptosis. Under pathological conditions of increased endothelial apoptosis, regeneration of injured endothelium is enhanced by circulating endothelial progenitor cells (EPC) [11]. Circulating EPC (bone marrow- or monocyte-derived) are inversely correlated with atherosclerosis risk factors and clinical outcomes, and therefore represent integrative biomarkers of vascular health [12].

Cardiovascular effects of natural dietary supplements such as rice bran are of growing interest [13]. Rice bran is a byproduct of rice milling which exerts lipid-lowering, antioxidant, anti-diabetic and anti-inflammatory activities due to the high content in phytochemicals such as γ-oryzanol (esters of ferulic acid and of triterpene alcohols or plant sterols), phytosterols and tocotrienols [14]. The use of rice bran by the food industry has been limited by the poor solubility of its components and by fast lipase degradation leading to rancidity. These weaknesses were overcome by the enzymatic extraction [15]. Rice bran and its by-products are able to prevent atherosclerosis plaque development and to protect from endothelial dysfunction by increasing cellular stress resistance [16, 17]. Although the

chemical properties of rice bran are known, the main components responsible for its effects as well as the mechanisms of action and its relevance for human biology remain unclear. We hypothesized that mitochondrial function is a crucial mediator of the effects of rice bran enzymatic extract (RBEE).

The aims of this study were to characterize the effects of RBEE on mitochondrial biogenesis and dynamics in relation to oxidative stress in the vasculature of ApoE-/- mice. We identified ferulic acid (FA) as the main compound present in the extract responsible for the actions observed. We measured FA and FA-derived metabolites accumulation in mice liver and kidney and its human bioavailability. Finally, we performed a human study evaluating the impact of FA consumption on peripheral blood mononuclear and endothelial progenitor cells.

2. Material and Methods

2.1 Rice bran enzymatic extract (RBEE)

Rice bran enzymatic extract was prepared and chemically characterized as previously described [15]. Briefly, raw rice bran was introduced in a bioreactor, pH (8.0) and temperature (60 °C) controlled. The extraction was performed with a hydrolytic trypsin- and chymotrypsin-like endoproteases mixture (Bioproteasa LA450, Biocon Española, Spain). Nutraceutical composition in RBEE is as follows: γ-oryzanol (8950 mg/kg), phytosterols (3553 mg/kg), tocotrienols (170 mg/kg) and tocopherols (93.4 mg/kg).

2.2 Animals and diets

All animal experiments were performed conforming the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Ethic Committee for Animal Experimentation of the University of Seville (Spain) (AGL2013-407791-P). Male apolipoprotein E knockout (ApoE-/-) mice on a C57BL/6J background and wild-type (C57BL/6J) mice were purchased from Charles River Laboratories (L'Abresle, France). At seven weeks of age, ApoE-/- mice were randomized to high-fat diet (HFD) (TD 88137, Teklad, Envigo, Madison, USA) containing 0.15% (w/w) cholesterol and 42% (% kcal) fat or HFD supplemented with 5% (w/w) of RBEE (HFD 5% RBEE) for 21 weeks. As non-atherosclerotic control, age-matched wild-type mice were kept on standard diet (STD) (2014, Teklad, Envigo, Madison, USA). At 28 weeks of age mice were sedated by an

intraperitoneal injection of ketamine/xylazine (100/10 mg/kg) and sacrificed by exsanguination.

2.3 Cell culture

Bovine aortic endothelial cells (BAEC; passages 3-6) were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin; Life Technologies), 4 mM L-glutamine and 10% fetal bovine serum (Gibco, Invitrogen) [18]. Cells were harvested for 24 hours and then cultured for 18 hours in the presence of 367.6 mg/l RBEE or one of its main bioactive components at the same concentration at which those are found in the dose of RBEE used: 5.5 µM ferulic acid (FA), 5.5 µM γ-oryzanol (OZ), 1.5 µM β-sitosterol (β-Sitos) or 0.12 µM γ-tocopherol (γ-Toco). Then, cells were co-incubated with 100 µM H_2O_2 for 4 additional hours, washed twice, lysed by sonication in the suitable buffer for the different assays performed and frozen at -80 °C until analysed.

2.4 Human study - Serum values

All human studies have been approved by the local ethics committee (Number 162/15) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All subjects provided written informed consent prior to their inclusion in the study.

Five non-smoking healthy volunteers (3 males and 2 females, mean age: 35.4 ± 3.7 years) consumed 500 mg FA (Source Naturals, Scotts Valley, USA) per day prior to breakfast and followed a balanced diet. On the first day, FA absorption was measured at the following times: 0 min, 30 min, 60 min, 180 min, 360 min and 24 hours. An additional tube was collected before FA consumption for serum lipid, glucose and liver enzymes measurement.

2.5 Human study - Isolation and treatment of peripheral blood mononuclear cells (PBMC)

To test the *in-vivo* effects of FA in humans, PBMC were isolated from 40 ml of blood before and after 15 days of treatment with FA by Ficoll density gradient centrifugation [18-20].

Furthermore, the effects of FA were tested *ex-vivo* in cultured PBMC from 5 untreated healthy volunteers. For each *ex-vivo* study, 1 million freshly isolated PBMC were plated in RPMI medium at 37 °C in 5% CO_2 atmosphere and treated with 5.5 µM FA or an equal volume of solvent (control condition) for 18 hours, starting after the isolation. Then, the cells were co-treated with 100 µM H_2O_2 alone or in the presence of FA for 4 additional hours to induce

oxidative stress and apoptosis. PBMC from the *in-vivo* study were directly treated with solvent or 100 µM H_2O_2 for 4 hours after the isolation, washed twice and used for the assays.

2.6 Determination of glutathione levels, aconitase activity and lipid peroxidation

Spectrophotometric measurement of reduced (GSH) and oxidized (GSSG) forms of glutathione was based on DTNB consumption and measured in aortic tissue from mice and BAEC as previously described [21]. Results are presented as GSH/GSSG ratio. Aconitase catalyzes the reaction of sodium citrate to isocitrate, using $NADP^+$ as electron acceptor. Aconitase activity from aortic homogenates was measured spectrophotometrically by registering the formation of NADPH as previously described [22] and is expressed as mU per mg protein. Malondialdehyde (MDA) quantification served as an indicator of lipid peroxidation and was carried out in aortic tissue and BAEC using the ALDetect™ Lipid Peroxidation Assay Kit following the manufacturer's recommendations. Results are expressed as nM malondialdehide (MDA) per mg protein [21].

2.7 NADPH oxidase activity assay

A lucigenin-enhanced luminescence assay was performed to determine basal and phorbol myristate acetate (PMA)-stimulated NADPH oxidase activity in human PBMC from the *in*-vivo study as reported before [19, 20].

2.8 Quantification of atherosclerotic plaques in the aorta

3 µm cross sections of 4% paraformaldehyde-fixed aortic arches were used to quantify atherosclerotic lesions after hematoxylin/eosin staining. Tissue recorded with an Olympus BX61 microscope under the same magnification (10x objective) and atherosclerotic plaque area was measured using ImageJ v1.45 software.

2.9 Western Blot

Immunoblots were carried out in aortic tissue and BAEC protein lysates as previously described [16] with the following primary antibodies: anti-peroxiredoxin III (1:500; Biomol-Enzo Life Sciences, Lörrach, Germany), anti-peroxiredoxin-SO_3 (1:500; AbFrontier, Seoul, Korea), anti-peroxiredoxin-I (1:500; Cell Signaling, Technology, Beverly, USA) anti-AMPKα2 (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany), anti-p-AMPKα (Thr172) (1:1000; Cell Signaling, Technology, Beverly, USA) or anti-β-actin (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) [16]. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4000) or donkey anti-goat IgG (1:4000) were used against the appropriate species of primary antibody. Immune complexes were visualized with the use of enhanced

chemiluminescence (ECL) and exposition to autoradiography films (GE Healthcare, Munich, Germany), which were developed in an Agfa Curix 60 system. Densitometric analyses of the resulting bands were performed using Image Studio Lite v.4.0.21 software (LI-COR, Nebraska, USA) and β-actin was used as internal control to verify equal protein loading in all blots.

2.10 Immunoprecipitation

Immunoprecipitation of PGC-1α was performed to assess its level of acetylation. 250 µg of aortic lysates were incubated overnight with PGC-1α antibody (1 µg antibody / 100 µg protein). Then, agarose beads were added and incubated in constant movement over 4 hours, washed and resuspended Laemmli loading buffer. Immunoprecipitates were analysed by immunoblotting using antibodies against acetylated lysine residues (AKL5C1) (1:100, Santa Cruz Biotechnology, Heidelberg, Germany) and PGC-1α (H-300) (1:500, Santa Cruz Biotechnology, Heidelberg, Germany).

2.11 qRT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine the expression of genes related to mitochondrial biogenesis and dynamics in in a StepOnePlus™ Real-Time PCR System, as previously reported [16]. Primer sequences for *Pgc-1α, Pgc-1β, Nrf-1, Nrf-2, Tfam*, *Mfn1, Mfn2, Opa1, Fis1* and *Beclin-1* and thermal cycler conditions are listed in **Suppl. Table 4**.

2.12 Ferulic acid metabolites determination by UHPLC

Ferulic acid and ferulic acid metabolites were measured in plasma, liver and kidney as previously described [23, 24]. Phenolic compounds were analysed by AcQuity Ultra-PerformanceTM liquid [matography and tandem mass spectrometry using AcQuity BEH C18 column equipped with a VanGuardTM Pre-Column AcQuity BEH C18. Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer equipped with a Z-spray electrospray interface. Ionization was achieved using electrospray (ESI) interface operating in the negative mode $[M-H]^-$ and data was acquired through selected reaction monitoring (SRM).

2.13 Measurement of PBMC apoptosis/necrosis by Annexin V / propidium iodide FACS

Flow cytometric assessment of Annexin V / propidium iodide staining was used to quantify apoptosis and necrosis of PBMC from the *in-vivo* and *ex-vivo* studies as described before [18, 20].

2.14 Measurement of oxidative stress by dihydroethidium (DHE) FACS and staining

For FACS analysis of oxidative stress in PBMC from the *ex-vivo* study, harvested cells were stained in 2 µM DHE solution and measured in a FACSCalibur instrument (Becton-Dickinson). Cell Quest software was used for quantification. In addition, PBMC were treated with FA and H_2O_2 as described before. The harvested cells were fixed with a 4% formaldehyde solution for 20 min, washed twice with water and resuspended in 2 µM DHE solution. After incubation for 30 min at 37 °C in 5% CO_2 atmosphere, 100 µl cell suspension was added in a cytospin apparatus and centrifugated for 5 minutes at 400 g. DHE+ cells were visualized by fluorescence microscopy.

2.15 Assessment of the number and function of endothelial progenitor cells (EPC)

To select for endothelial progenitor cells (EPC), PBMC from the ferulic acid *in-vivo* treatment study were isolated as described above and cultured on fibronectin-coated culture dishes in endothelial basal medium (EBM; Lonza) with supplements (1 µg/mL hydrocortisone, 3 µg/mL bovine brain extract, 30 µg/mL gentamicin, 50 µg/mL amphotericin B, 10 µg/mL human endothelial growth factor and 20% fetal calf serum) [19].

To analyze EPC differentiation, EPC were identified by the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (DiLDL) and binding to FITC-labeled Ulex europaeus agglutinin I (lectin). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Colony-forming capability is a key measure of EPC proliferative capacity. 48 h after Ficoll isolation and seeding, non-adherent cells in the supernatant were collected and 1 million cells were transferred to fibronectin-coated 24-wells and cultured in EBM with supplements for 7 more days. Colonies, defined as clusters of more than 15 viable cells, were counted under a light microscope.

SDF-1-induced EPC migration was measured by the modified Boyden chamber assay in HTS Fluoroblocks with 8 µm pores using 100 ng/mL SDF-1 to induce migration. After incubation at 37 °C for 24 h, the filters were carefully washed, cells fixed and incubated with diLDL as described above. DiLDL-positive EPC that had migrated to the lower surface of the filter in response to SDF-1 were quantified using fluorescence microscopy (400 X magnification).

2.16 Statistical analysis

Values were reported as mean ± SEM, and significance of the differences between mean values was determined by one way analysis of variance (ANOVA), combined with the Bonferroni's multiple comparison test, or by Wilcoxon test where appropriate. A p-value of <0.05 was considered statistically significant.

3. Results

3.1 RBEE diet supplementation reduces oxidative stress in mice aorta.

As control experiments, previously published work was repeated to confirm the induction of atherosclerotic plaque formation by high-fat diet (HFD) in ApoE-/- in the present study. Atherogenesis was potently prevented by supplementation of the HFD with 5% RBEE (**Fig. 1A-C**; HFD: 690.4 ±135.8; HFD 5% RBEE: 263.8±148.3; p<0.05) [16].

The effects on atherogenesis were paralleled by the effects on regulators of oxidative stress. HFD reduced the ratio of glutathione (GSH) to glutathione disulfide (GSSG) (p<0.01), which was restored by supplementation of the HFD with RBEE (p<0.05, **Fig. 1D**). Peroxiredoxins are a ubiquitous family of antioxidant enzymes. The increased oxidation of peroxiredoxin (Prx-SO_3) induced by HFD in ApoE-/- mice (p<0.05) was counteracted by RBEE diet supplementation (p<0.05, **Fig. 1E**). The expression level of total (Prx-I; data not shown) and mitochondrial peroxiredoxin (Prx-III) remained unchanged in ApoE-/- mice fed HFD or HFD-supplemented diets (**Fig. 1F**). The aconitase enzyme catalyzes the interconversion of citrate and isocitrate in the Krebs cycle. HFD-fed ApoE-/- mice showed reduced aconitase activity (**Fig. 1G**, p<0.01), however, this was not prevented by RBEE. Lipid peroxidation as measured by malondialdehyde (MDA) was induced by HFD (**Fig. 1H**, p<0.05) and fully prevented by RBEE diet supplementation (p<0.05).

3.2 Mitochondrial biogenesis and dynamics are restored by RBEE treatment.

Importantly, high-fed diet resulted in lower vascular mRNA expression of mitochondrial markers of biogenesis (*Pgc-1α, Pgc-1β and Nrf-1),* fusion *(Mfn1* and *Mfn2),* fission *(Fis1)* and mitophagy *(Beclin-1*) in ApoE-/- mice (**Fig. 2**, p<0.05). RBEE diet supplementation restored the expression of *Pgc-1α, Pgc-1β, Nrf-1, Mfn1, Mfn2, Fis1 and Beclin-1* to the control level expressed in wild-type mice (p<0.05). *Nrf-2, Tfam* and *Opa1* mRNA levels remained unchanged in ApoE-/- mice and were not regulated by RBEE treatment.

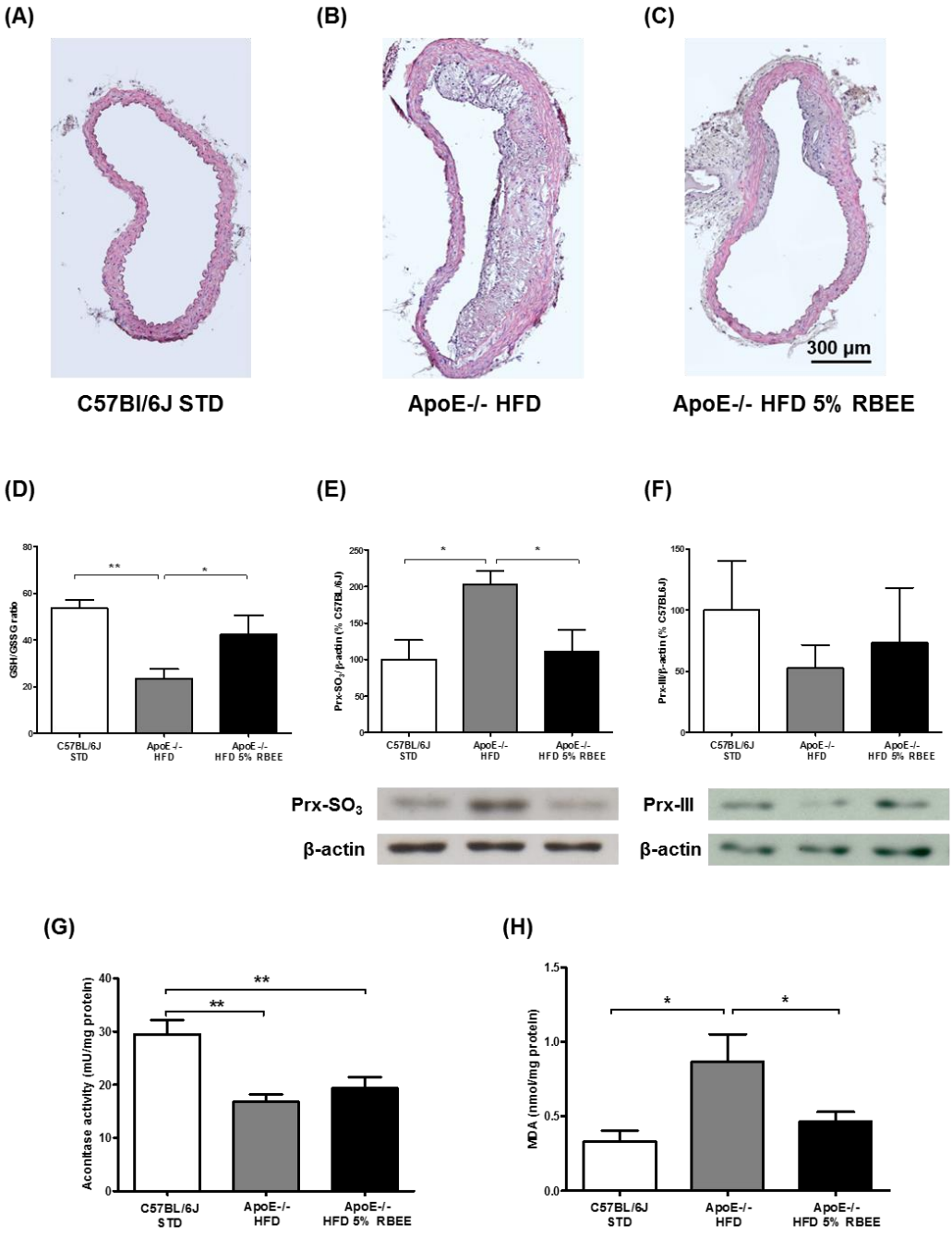

Figure 1: RBEE reduces atherosclerosis and oxidative stress in the aorta of ApoE-/- mice on high-fat diet.

Markers of oxidative stress were measured in the aorta of wild-type mice fed standard diet (C57BL/6J STD) or ApoE-/- mice fed high-fat diet (ApoE-/- HFD) or high-fat diet supplemented with 5% rice bran enzymatic extract (ApoE-/- HFD 5% RBEE) for 21 weeks. (**A-C**) Representative pictures of hematoxylin/eosin staining of atherosclerotic plaques in the aorta ascendens. (**D**) Ratio of reduced (GSH) to oxidized (GSSG) glutathione. Protein expression of peroxiredoxin-SO3 (**E**) and peroxiredoxin-III (**F**) were determined by Western blot using β-actin as loading control. (**G**) Aconitase activity. (**H**) Malondialdehyde (MDA) served as indicator of lipid peroxidation. Values are expressed as mean ± SEM of 6 animals. *($p<0.05$) and **($p<0.01$).

	C57BL/6J STD	ApoE-/- HFD	ApoE-/- HFD 5% RBEE
Pgc-1α	100 ± 15.3	46.7 ± 9.0 *	115.6 ± 19.7 +
Pgc-1β	100 ± 34.3	35.8 ± 8.8 *	85.4 ± 22.0 +
Nrf-1	100 ± 30.6	38.7 ± 13.6 *	151.3 ± 22.6 * +
Nrf-2	100 ± 22.6	64.7 ± 4.0	61.5 ± 3.8
Tfam	100 ± 44.0	82.1 ± 17.0	93.7 ± 39.7
Mfn1	100 ± 15.7	40.6 ± 8.5 *	100.7 ± 19.6 +
Mfn2	100 ± 23.3	38.9 ± 8.1 *	105.0 ± 17.3 +
Opa1	100 ± 23.2	81.5 ± 11.1	109.1 ± 20.7
Fis1	100 ± 15.0	58.1 ± 8.7 *	107.1 ± 18.0 +
Beclin-1	100 ± 16.7	49.0 ± 24.5 *	115.0 ± 13.6 +

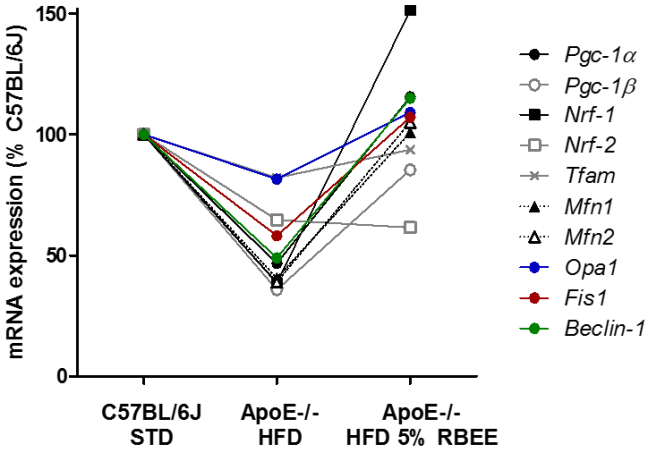

Figure 2: RBEE improves mitochondrial biogenesis and dynamics in the vascular wall of ApoE-/- mice.
Markers of mitochondrial biogenesis (Pgc-1α, Pgc-1β, Nrf-1, Nrf-2 and Tfam) and dynamics (Mfn1, Mfn2, Opa1, Fis1 and Beclin-1) in the aorta of wild-type (C57BL/6J STD) and ApoE-/- mice fed with HFD +/- RBEE for 21 weeks were determined by quantitative real-time PCR and normalized to 18s. C57BL/6J mice were used as the comparator group, and % values are mean ± SEM of 6 animals. Significant differences are indicated by *(p<0.05) vs C57BL/6J or +(p<0.05) vs ApoE-/- HFD.

3.3 RBEE activates upstream regulators of mitochondrial biogenesis.

ApoE-/- mice showed increased expression of AMPK (**Fig. 3A**, p<0.05). However, only those mice receiving RBEE supplementation also had an increase in AMPK activity as demonstrated by increased phosphorylation (**Fig. 3B**, p<0.05). Peroxisome proliferative activated receptor-α coactivator 1 (PGC-1α) is a master regulator of mitochondrial

biogenesis and is activated by p-AMPK and *Sirt1*. *Sirt1* mRNA expression remained unchanged (**Fig. 3C**). However, acetyl-lysine residues in PGC-1α precipitates were higher in ApoE-/- mice on HFD (p<0.01), showing decreased PGC-1α activity. Acetylation of PGC-1α lysine residues was prevented by RBEE supplementation (p<0.05), indicating higher PGC-1α activity (**Fig. 3D**).

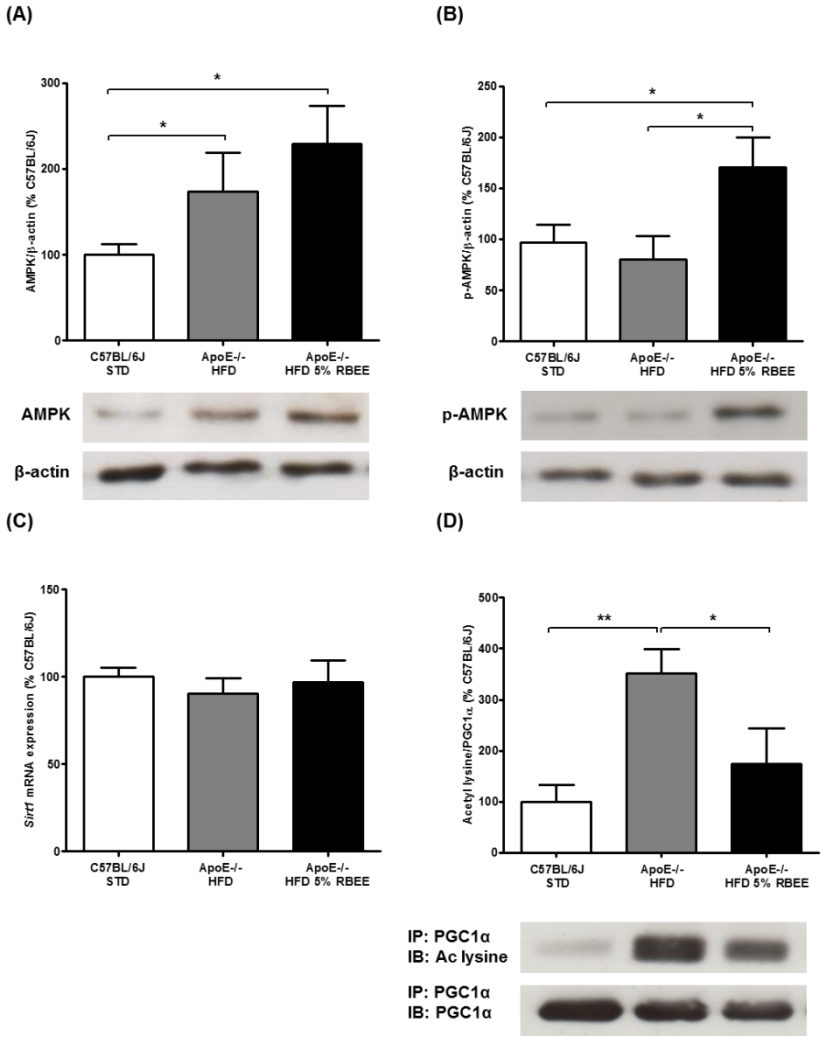

Figure 3: RBEE activates upstream mitochondrial regulators in the aorta of ApoE-/- mice on high-fat diet.

Inductors of PGC-1α deacetylation (p-AMPK and Sirt1) were measured in the aorta of wild-type and ApoE-/- mice fed with HFD +/- RBEE for 21 weeks. Protein expression of total AMPK (**A**) and phosphorylated-AMPK (p-AMPK) (**B**) were determined by Western blots using β-actin as loading control. Sirt1 mRNA level was determined by quantitative real-time PCR and normalized to 18s (**C**). PGC-1α was immunoprecipitated from aortic lysates and before immunoblotting for acetylated lysine and PGC-1α (**D**). Results are expressed as mean ± SEM of 6 animals. *(p<0.05) and **(p<0.01).

3.4 Ferulic acid is the main responsible component for RBEE activities.

In-vitro studies were performed in BAEC in order to identify the components of RBEE which confer mitochondrial-protective activities. RBEE and its known main components were applied at their respective concentrations present in the enzymatic extract. Stimulation with 100 µM H_2O_2 induced oxidative stress as shown by reduced GSH/GSSG ratio (**Fig. 4A**, $p<0.05$) and higher MDA levels (**Fig. 4B**, $p<0.001$). Incubation with RBEE or FA counteracted this effect (**Fig. 4AB**). γ-oryzanol (OZ) prevented GSH oxidation, but did not reduce lipid peroxidation. γ-tocotrienol (γ-Toco) had the opposite effect; it prevented lipid peroxidation ($p<0.05$) but did not modify GSH/GSSG ratio. Incubation with β-sitosterol (β-Sitos) had no effect on H_2O_2-induced oxidative stress in endothelial cells.

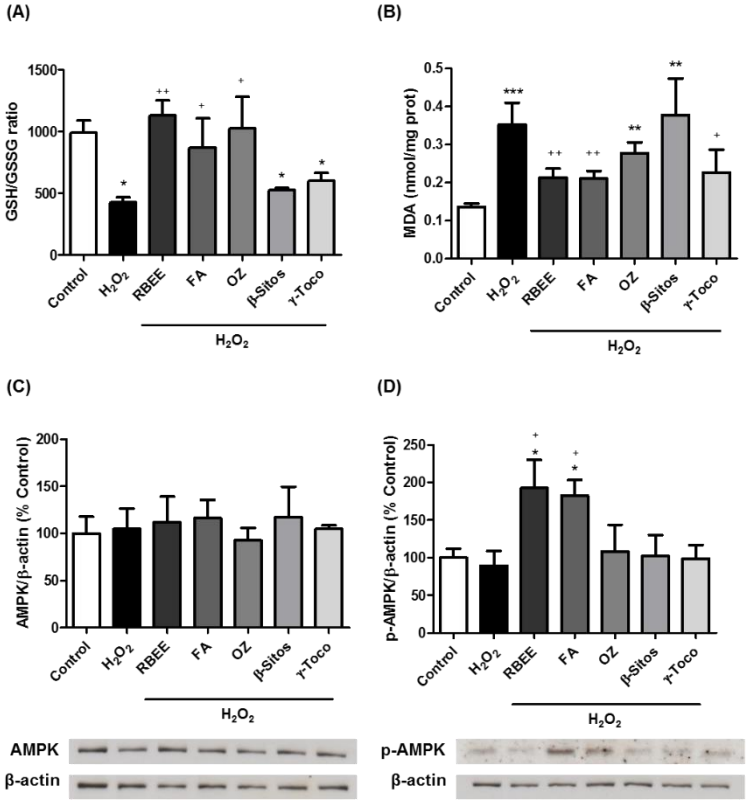

Figure 4: Cellular effects of RBEE and its major bioactive compounds in bovine aortic endothelial cells

Bovine aortic endothelial cells (BAEC) were treated with 100 µM H_2O_2 alone or in presence of rice bran enzymatic extract (RBEE, 367.6 mg/L) or its major bioactive compounds: ferulic acid (FA, 5.5 µM), γ-oryzanol (OZ, 5.5 µM), β-sitosterol (β-Sitos, 1.5 µM) or γ-tocotrienol (γ-Toco, 0.12 µM) and markers of oxidative stress and metabolism were measured. **(A)** Reduced (GSH) to oxidized (GSSG) glutathione ratio. **(B)** Malondialdehyde (MDA) served as indicator of lipid peroxidation. Protein expression of total AMPK **(C)** and phosphorylated-AMPK (p-AMPK) **(D)** were determined by Western blot using β-actin as loading control. Results are expressed as mean ± SEM of 3 - 6 independent experiments. *($p<0.05$), **($p<0.01$) and ***($p<0.001$) vs Control or $^{+}$($p<0.05$) and $^{++}$($p<0.01$) vs H_2O_2.

Incubation with 100 μM H_2O_2, alone or in combination with RBEE or its bioactive compounds did not modify the expression of total AMPK (**Fig. 4C**). However, RBEE and FA induced AMPK activation by phosphorylation (**Fig. 4D**, $p<0.05$). γ-oryzanol, β-Sitos and γ-tocotrienol did not modify p-AMPK expression. Taken together, these data identify FA as the main active compound in RBEE with regard to the prevention of endothelial oxidative stress and AMPK activation.

3.5 Ferulic acid metabolites are accumulated in mice liver and kidney after chronic RBEE consumption.

Ferulic acid (FA) concentration and FA metabolites were measured in mice liver, kidney and fasting plasma after 21 weeks of RBEE supplemented diet consumption. After 12 hours of overnight fasting, neither FA nor any of its metabolites were found in plasma (data not shown). However, FA and FA-derived metabolites were present in the liver and the kidney. Compared to C56BL/6 mice, the 21-week period of HFD consumption in ApoE-/- mice *per se* induced an increase in FA and phenolic compounds in liver and kidney (**Suppl. Table 1**). In mice supplemented with RBEE, these levels were further increased, confirming FA absorption and tissue retention of its metabolites. Catechol sulphate was the most abundant compound in both tissues. However, although most of the metabolites were found in both tissues, hydroxyphenylacetic acid sulphate, dihydroxyphenylacetic acid and dihydroxyphenypropionic acid were only detected in liver, while dihydroxyphenylacetic acid glucuronide, hydroxyphenypropionic acid, vanillic acid sulphate, catechol glucuronide, hydroxybenzoic acid and hydroxybenzoic acid glucuronide were only identified in kidney.

3.6 Ferulic acid absorption kinetics in human plasma.

FA and FA-derived metabolites were measured in human plasma after acute consumption of 500 mg ferulic acid in five healthy volunteers. Twenty-one phenolic metabolites were identified (**Suppl. Table 2**). The most abundant metabolites were FA, FA sulphate, FA glucuronide, hydroxyphenylacetic acid sulphate and dihydroxyphenylacetic acid. FA, FA sulphate and FA glucuronide showed typical first order absorption curves, with one absorption peak between 1 and 3 hours followed by a clearance period which nearly reached the baseline after 24 hours ($t_{1/2\beta}$: 1.3 ± 0.1 h). However, hydroxyphenylacetic acid sulphate and dihydroxyphenylacetic acid plasma concentrations increased with time over the 24 hours period, reaching a plateau after 6 hours. When the AUC of the absorption curves were calculated, 18.3±6.0 mg free and non-metabolized FA and 493.5±27.2 mg of FA equivalents derived from metabolites were found.

Serum lipids (total cholesterol, LDL-C, HDL-C, triglycerides), glucose, liver enzymes (aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase), bilirubin and C-reactive protein were quantified before and after FA consumption. All these parameters remained unchanged (**Suppl. Table 3**).

3.7 Ferulic acid prevents oxidative stress in human PBMC.

Human PBMC were isolated before and after consumption of 500 mg FA per day for 15 days and were used to investigate oxidative stress (n=5). NADPHox activity was lower after FA consumption both, in basal conditions ($p<0.05$) and after induction with PMA ($p<0.05$) (**Fig. 5A**). To confirm this result, the product of NADPHox, superoxide, was measured by DHE FACS in human PBMC treated *ex-vivo* with 100 µM H_2O_2 alone or in presence of 5.5 µM FA. H_2O_2 induced higher superoxide production ($p<0.05$) which was abolished by FA co-treatment ($p<0.05$, **Fig. 5B-E**). Then, PBMC resistance to H_2O_2-induced apoptosis and necrosis after 15 days FA consumption was assessed. Incubation with H_2O_2 induced 4.7-fold increased apoptosis ($p<0.01$) and 3.7-fold increased necrosis ($p<0.01$, data not shown) (**Fig. 5G**) and was prevented by FA consumption ($p<0.05$). The prevention of H_2O_2-induced PBMC apoptosis was confirmed by *ex-vivo* incubation of PBMC with 100 µM H_2O_2 alone or in presence of 5.5 µM FA. The 2-fold increase in apoptotic PBMC induced by H_2O_2 ($p<0.05$) was fully ameliorated by co-incubation with FA (**Fig. 5G**).

3.8 Mitochondrial marker expression in PBMC and EPC differentiation and function are improved after ferulic acid consumption.

Human PBMC and EPC before and after consumption of 500 mg FA per day for 15 days were isolated for measuring expression of markers of mitochondrial biogenesis, fusion and fission, as well as EPC number and function. In human PBMC, we observed a two-fold increase in mRNA expression of marker of mitochondrial biogenesis PGC-1α ($p<0.01$; **Fig. 6A**) and fusion MFN1 ($p<0.05$; **Fig. 6B**). mRNA expression of FIS1 remained unchanged (**Fig. 6C**).

EPC function before and after FA treatment was evaluated by measuring differentiation, proliferation and migration. Ferulic acid induced an increased number of EPC in culture ($p<0.05$; **Fig. 6D**) as well as enhanced EPC proliferation ($p<0.05$; **Fig. 6E**). EPC migration was not altered (**Fig. 6F**).

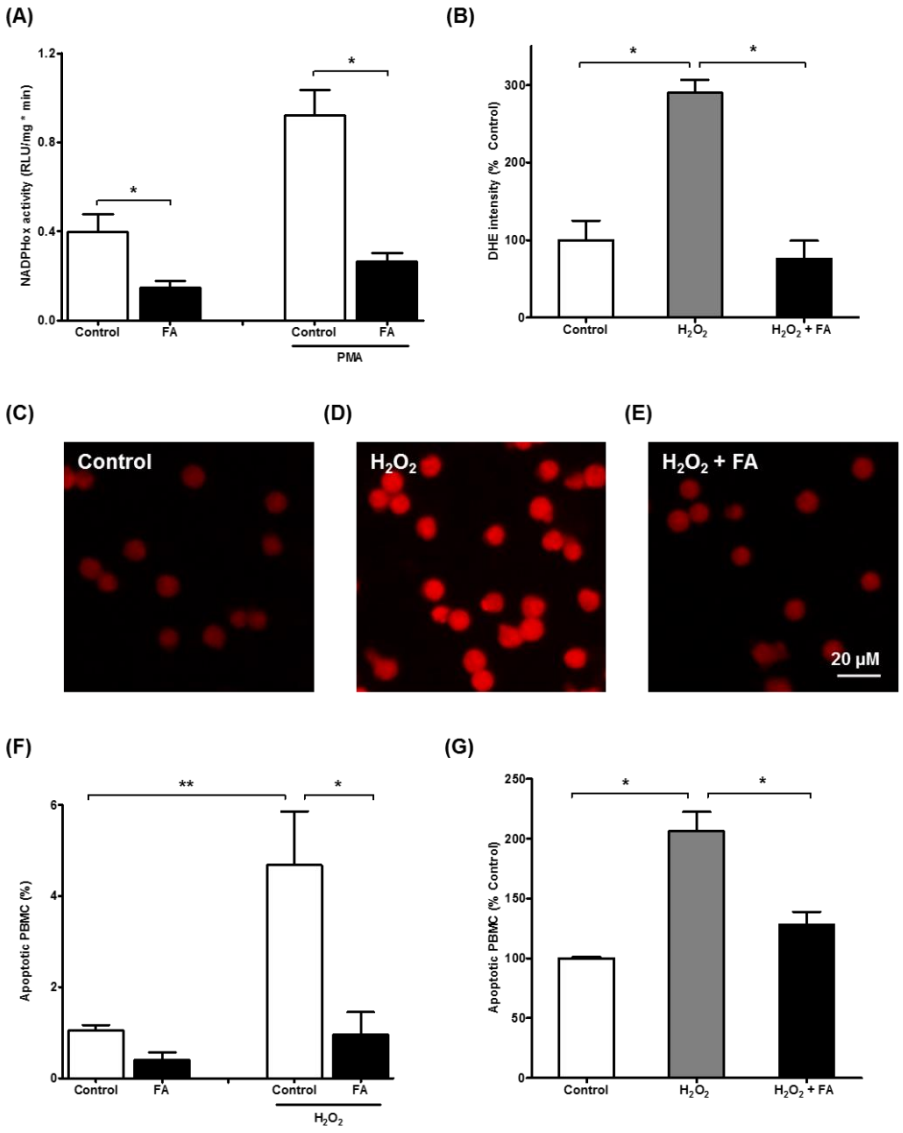

Figure 5: Ferulic acid treatment reduces oxidative and apoptosis in human PBMC *in-vivo* and *ex-vivo*.

Human volunteers consumed 500 mg ferulic acid (FA) per day for 15 days and PBMC were isolated for analyses. **(A)** NADPH oxidase (NADPHox) activity at baseline (control condition) and after 15 days of ferulic acid consumption. Basal activity and PMA-induced activity of NADPHox are expressed as relative luminescence units (RLU) per mg of protein and minute. **(B)** *Ex-vivo* treatment of human PBMC with 100 µM H_2O_2 alone or in presence of 5.5 µM ferulic acid (FA) was used to measure superoxide production by dihydroethidium luminescence (DHE) by FACS. **(C-E)** Representative images of DHE-stained PBMC in culture. **(F)** Apoptosis of human PBMC was measured by FACS (Annexin V / Propidium Iodide) in control conditions and after 15 days of ferulic acid consumption in human PBMC untreated or stimulated with 100 µM H_2O_2. **(G)** Ferulic acid-induced prevention of apoptosis was confirmed by *ex-vivo* treatment of human PBMC with 100 µM H_2O_2 alone or in presence of 5.5 µM ferulic acid. Results are expressed as mean ± SEM of 5 subjects (*in-vivo*) or 5 experiments (*ex-vivo*), respectively. Significant differences are indicated by *($p<0.05$) and **($p<0.01$).

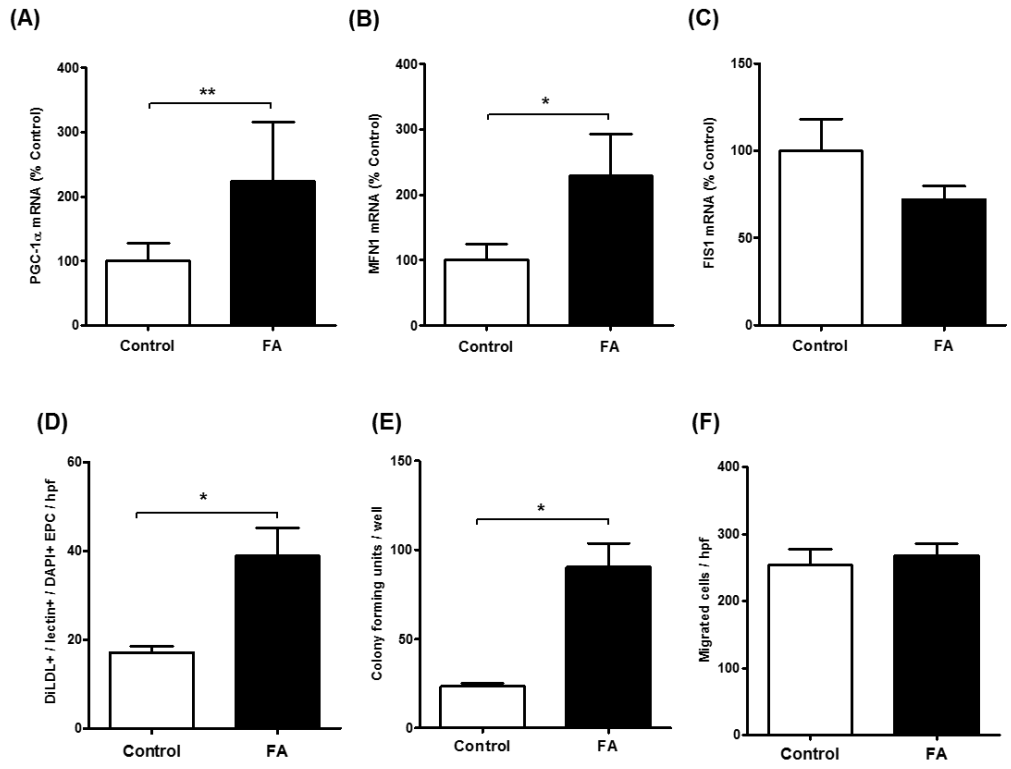

Figure 6: Ferulic acid treatment up-regulates mitochondrial markers in PBMC and improves endothelial progenitor cell number and function in humans
mRNA levels of PGC-1α **(A)**, MFN1 **(B)** and FIS1 **(C)** in human PBMC before (Control) and after 15 days of ferulic acid consumption (FA) were determined by quantitative real-time PCR and normalized to 18s. Endothelial progenitor cell (EPC) differentiation was measured by DiLDL-lectin-DAPI triple staining and quantified by immunofluorescence microscopy **(D)**. EPC proliferation was quantified by counting EPC colonies by light microscopy (Hill assay) **(E)** and EPC migration **(F)** was determined by a modified Boyden chamber assay and quantified by fluorescence microscopy. Results are expressed as mean ± SEM of 5 subjects. *($p<0.05$) and **($p<0.01$).

4. Discussion

In our study, ferulic acid was identified as the main compound responsible for the vasculo-protective effects of rice bran. Dietary supplementation with ferulic acid alone or as a component of rice bran enzymatic extract improved mitochondrial biogenesis and dynamics and reduced oxidative stress in mouse aorta and human monocytes.

Vascular oxidative stress plays a pivotal role for the development of atherosclerosis and other cardiovascular diseases [1]. Our data show that high-fat diet induces a variety of markers of oxidative stress in the vessel wall including the reduction of the GSH/GSSG ratio, oxidation of the anti-oxidative peroxiredoxin, and lipid peroxidation. Diet supplementation

with RBEE was able to potently prevent these components of vascular oxidative stress. Rice bran components such as γ-oryzanol, ferulic acid and tocotrienols protect biological molecules against oxidative modifications due to direct ROS scavenger activity and regulation of pro-oxidant and antioxidant enzymes [14, 25]. Here, these data are extended to vascular biology and mitochondrial function.

Studies on vascular damage induced by shear stress demonstrated superoxide production [26] and parallel effects on aconitase activity, a sensitive biomarker of acute oxidative stress [27, 28]. Aconitase activity was significantly reduced in atherosclerotic ApoE-/- mice on high-fat diet, but RBEE did not change aconitase activity in contrast to the mediators mentioned above. These data demonstrate marked long-term anti-oxidative effects of RBEE, while more short-term effects appear to by masked by the pro-oxidant vascular environment present in ApoE-/- mice.

Mitochondrial biogenesis and dynamics processes represent a tightly regulated interplay of cellular and molecular processes that eventually shape metabolic and bioenergetic cell function [7]. Importantly, the experiments show that RBEE supplementation potently preserved the expression of biogenesis markers *Pgc-1α, Pgc-1β* and *Nrf-1*, which were down-regulated in HFD-fed animals. Parallel effects were observed with regard to PGC-1α activity as assessed by quantification of PGC-1α acetylation, and on Fis1 and Beclin-1 that have been connected to increased oxidative damage and apoptosis. While mitochondrial fusion is crucial to enlarge mitochondria in the early steps of biogenesis, fission allows dysfunctional mitochondria to be eliminated by autophagy. The data reveal that high fat diet-induced a reduction of both fusion (*Mfn1* and *Mfn2*) as well as fission (*Fis1*) mediators which were potently prevented by RBEE diet supplementation. Impaired mitochondrial recycling and programmed mitophagy may initiate a vicious cycle where the accumulation of damaged mtDNA leads to impaired fusion, fission and mitophagy mechanisms themselves [29].

Although we did not observe changes in *Sirt1* mRNA expression, AMPK expression and AMPK activation were up-regulated by RBEE *in-vitro* and *in-vivo*. AMPK is known to increase PGC-1α activity and expression and is associated to greater SIRT1 activity mediated by post-transcriptional phosphorylation and by increasing NAD/NADH ratio [8, 30]. Taken together, the study shows quantitatively relevant and consistent impairment of markers of mitochondrial function and morphology induced by high-fat diet that is potently prevented by RBEE.

Most of the described effects of rice bran have been attributed to γ-oryzanol, which is a mixture of ferulic esters of triterpenic alcohols and sterols. Steryl ferulates cannot pass through

the intestinal barrier [31]. Steryl ferulates are hydrolyzed to ferulic acid that can be absorbed and mediate systemic effects. Accordingly, the FA and its metabolites are present in the liver and the kidney of mice treated with RBEE. Ferulic acid and metabolites are also found in lower concentration in tissues of HFD-fed control animals since this polyphenol is common in chow diet containing carbohydrates from wheat or corn. Consistent with the short half-life of around 3 hours reported in rats [32, 33], FA was not found in the murine plasma 12 hour post feeding.

The studies in cultured endothelial cells confirmed the antioxidant activity of RBEE and identified that γ-oryzanol and ferulic acid but not β-sitosterol or γ-tocotrienol mimicked the cellular antioxidative effects of RBEE. Therefore, these results confirm that the effects of γ-oryzanol are mediated by its ferulic acid moiety [34]. Treatment with ferulic acid directly also fully reproduced the effects of RBEE including the effects on AMPK phosphorylation.

Ferulic acid kinetics were previously studied in rats but are yet unknown in human [23, 32, 33, 35, 36]. To study the relevance of the cell culture and the mouse experiments in humans, the plasma pharmacokinetics of ferulic acid-derived phenolic compounds were quantitated by UHPLC in healthy volunteers 1, 3, 6 and 24 hours after consumption of 500 mg of ferulic acid. The human data show efficient absorption, the whole dose consumed was found in plasma as free ferulic acid or as ferulic acid-derived molecules. The plasma half-life of elimination was short ($t_{1/2\beta}$: 1.3 ± 0.1 h). However, we detected twenty-one phenolic metabolites in the plasma. Some metabolites showed typical first order absorption curves. Others, such as hydroxyphenylacetic acid sulphate and dihydroxyphenylacetic acid, increased with time over the 24 hour period. The calculated AUC corresponds to a significant plasma concentration of ferulic acid metabolites (~18 mg free and non-metabolized FA and ~490 mg of FA equivalents). Most of the derived metabolites have been reported to exert a similar antioxidant capacity compared to ferulic acid [37]. Taken together, these data strongly suggest ferulic acid as the main mediator of the vascular effects of rice bran extract. The pharmacologic studies show plausible plasma and tissue concentrations consistent with meaningful effects after dietary consumption.

Based on these data, human volunteers were provided with ferulic acid supplements to test the biologic effect. After the 15 day treatment, freshly isolated PBMC showed lower NADPH oxidase activity and H_2O_2-induced apoptosis and necrosis. These data were confirmed by *in-vitro* treatment of human PBMC with ferulic acid. These data demonstrate the anti-oxidative effect of ferulic acid in humans and are in agreement with cell culture data on ferulic acid-mediated NADPH oxidase inhibition in VSMC and findings in spontaneously hypertensive rats [35, 36, 38].

Monocyte-derived EPC play a significant role in regeneration of damaged blood vessels and are related to a lower risk of atherosclerosis development. After 15 days of ferulic acid consumption, we observed higher EPC differentiation and proliferation in human samples. These data are in agreement with observations reported in rats and humans after consumption of polyphenol-rich foods [39-43]. Circulating EPC have been proposed as biomarkers for cardiovascular outcomes [12], therefore the observed improvement of EPC function after ferulic acid treatment is consistent with enhanced cardiovascular protection.

In conclusion, the study shows the potential of dietary rice bran supplementation to reduce oxidative stress and to preserve mitochondrial biogenesis and dynamics. The effects are mediated by AMPK and are a new mechanism to explain reduced atherosclerotic plaque development in RBEE-fed ApoE-/- mice. Ferulic acid was identified as the main bioactive component of RBEE responsible for these vaso-protective actions. This is the first study ferulic acid effects on vascular human cells. FA consumption improved PBMC ROS production, enhanced the resistance against H_2O_2-induced apoptosis and protected mitochondrial biogenesis and dynamics as well as EPC function.

Taken together, these findings provide novel insight into the effects of diets on the pathophysiology of atherogenesis and provide the mechanistic basis for further testing the vascular effects of ferulic acid in clinical studies.

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7. Conflict of interest:

None

8. References

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Supplementary Table 1a: Ferulic acid metabolites concentration in ApoE-/- mice liver.
Ferulic acid-derived metabolites after RBEE treatment were determined in the liver of wild-type and ApoE-/- mice fed with HFD +/- RBEE for 21 weeks by UHPLC. Concentrations of metabolites are expressed as nmol/g of fresh tissue (mean ± SEM of 10 animals). *($p<0.05$) *vs* C57BL/6J or $^{+}$($p<0.05$) *vs* ApoE-/- HFD. n.d. indicates non-detected compounds.

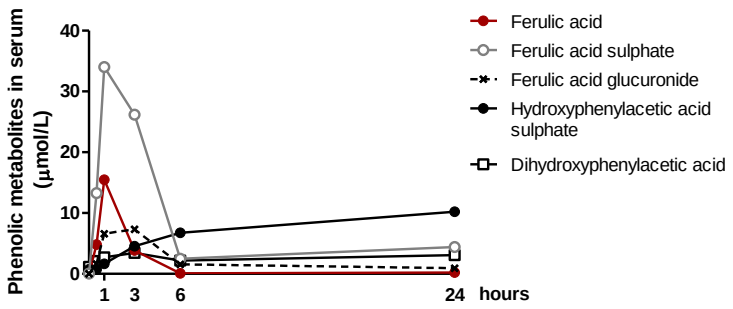
Phenolic compound	LIVER		
	C57BL/6J STD	ApoE-/- HFD	ApoE-/- HFD 5% RBEE
Ferulic acid	n.d.	11.24 ± 1.12 *	25.84 ± 3.73 * +
Isoferulic acid	n.d.	6.77 ± 0.49*	8.78 ± 1.49 *
Ferulic acid sulphate	n.d.	10.04 ± 0.61 *	31.87 ± 3.4 * +
Ferulic acid glucuronide	n.d.	4.97 ± 0.34 *	16.65 ± 2.00 * +
Dihydroferulic acid	n.d.	18.01 ± 1.83 *	30.75 ± 4.52 * +
Dihydroferulic acid sulphate	n.d.	33.13 ± 4.38 *	38.90 ± 16.38 *
Dihydroferulic acid glucuronide	n.d.	2.64 ± 1.51*	4.59 ± 0.53 * +
Caffeic acid sulphate	3.31 ± 0.12	4.11 ± 0.18 *	7.23 ± 0.40 * +
Caffeic acid glucuronide	n.d.	n.d.	3.31 ± 0.15 * +
Coumaric acid sulphate	n.d.	3.07 ± 0.18 *	11.27 ± 0.87 * +
Coumaric acid glucuronide	n.d.	0.29 ± 0.03 *	0.93 ± 0.19 * +
Phenylacetic acid	n.d.	66.82 ± 6.30 *	113.4 ± 8.71 * +
p-hydroxyphenylacetic acid	0.31 ± 0.24	55.22 ± 3.50 *	76.67 ± 3.82 * +
m-hydroxyphenylacetic acid	n.d.	7.69 ± 1.58 *	15.11 ± 1.94 * +
o-hydroxyphenylacetic acid	13.72 ± 3.65	10.38 ± 4.03	48.54 ± 6.83 * +
Hydroxyphenylacetic acid sulphate	8.52 ± 2.85	4.49 ± 1.56	17.99 ± 1.70 * +
Hydroxyphenylacetic acid glucuronide	n.d.	0.86 ± 0.25 *	10.42 ± 1.42 * +
Dihydroxyphenylacetic acid	n.d.	n.d.	13.98 ± 1.81 * +
Dihydroxyphenylacetic acid sulphate	n.d.	21.68 ± 3.14 *	67.76 ± 5.65 * +
Dihydroxyphenylacetic acid glucuronide	n.d.	n.d.	n.d.
Phenylpropionic acid	n.d.	8.75 ± 2.78 *	39.71 ± 6.27 * +
Hydroxyphenylpropionic acid	n.d.	n.d.	n.d.
Hydroxyphenylpropionic acid sulphate	n.d.	101.2 ± 6.30 *	261.1 ± 30.78 * +
Hydroxyphenylpropionic acid glucuronide	n.d.	n.d.	2.30 ± 0.84 * +
Dihydroxyphenylpropionic acid	n.d.	4.91 ± 0.22 *	7.26 ± 0.64 * +
Dihydroxyphenylpropionic acid sulphate	3.29 ± 0.12	4.73 ± 0.82	5.59 ± 0.37 *
Dihydroxyphenylpropionic acid glucuronide	n.d.	2.87 ± 0.12 *	3.59 ± 0.14 * +
Vanillic acid	n.d.	1.79 ± 0.71 *	5.99 ± 1.08 * +
Vanillic acid sulphate	n.d.	n.d.	n.d.
Homovanillic acid sulphate	n.d.	1.62 ± 0.44 *	3.10 ± 0.77 *
Hippuric acid	6.28 ± 0.19	15.88 ± 7.27	101.16 ± 6.43 * +
Catechol sulphate	n.d.	701.7 ± 37.74 *	1608 ± 158.2 * +
Catechol glucuronide	n.d.	n.d.	n.d.
Methyl catechol sulphate	n.d.	633.30 ± 34.84 *	1235 ± 99.3 * +
Methyl catechol glucuronide	n.d.	37.37 ± 3.05 *	80.76 ± 11.88 * +
p-hydroxybenzoic acid	0.68 ± 0.14	1.92 ± 1.11	14.65 ± 2.88 * +
Hydroxybenzoic acid	n.d.	n.d.	n.d.
Hydroxybenzoic acid sulphate	n.d.	3.97 ± 0.44 *	10.03 ± 0.84 * +
Hydroxybenzoic acid glucuronide	n.d.	n.d.	n.d.

Supplementary Table 1b: Ferulic acid metabolites concentration in ApoE-/- mice kidney.
Ferulic acid-derived metabolites after RBEE treatment were determined in the kidney of wild-type and ApoE-/- mice fed with HFD +/- RBEE for 21 weeks by UHPLC. Concentrations of metabolites are expressed as nmol/g of fresh tissue (mean ± SEM of 10 animals). *($p<0.05$) *vs* C57BL/6J or $^{+}$($p<0.05$) *vs* ApoE-/- HFD. n.d. indicates non-detected compounds.

Phenolic compound	KIDNEY		
	C57BL/6J STD	ApoE-/- HFD	ApoE-/- HFD 5% RBEE
Ferulic acid	n.d.	9.70 ± 0.96 *	25.51 ± 5.07 * $^{+}$
Isoferulic acid	n.d.	7.67 ± 0.37 *	19.07 ± 2.43 * $^{+}$
Ferulic acid sulphate	n.d.	21.97 ± 6.15 *	44.19 ± 7.86 * $^{+}$
Ferulic acid glucuronide	n.d.	11.26 ± 1.53 *	16.38 ± 3.03 *
Dihydroferulic acid	n.d.	19.72 ± 0.91 *	65.57 ± 12.49 * $^{+}$
Dihydroferulic acid sulphate	n.d.	16.69 ± 2.99 *	423.3 ± 80.47 * $^{+}$
Dihydroferulic acid glucuronide	n.d.	0.32 ± 0.13 *	9.73 ± 1.62 * $^{+}$
Caffeic acid sulphate	1.74 ± 0.04	2.19 ± 0.11 *	4.73 ± 0.46 * $^{+}$
Caffeic acid glucuronide	n.d.	n.d.	3.34 ± 0.22 * $^{+}$
Coumaric acid sulphate	n.d.	5.63 ± 1.22 *	9.80 ± 1.10 * $^{+}$
Coumaric acid glucuronide	n.d.	n.d.	0.46 ± 0.16 * $^{+}$
Phenylacetic acid	n.d.	9.12 ± 6.52 *	188.1 ± 35.83 * $^{+}$
p-hydroxyphenylacetic acid	n.d.	10.27 ± 0.16 *	73.42 ± 3.54 * $^{+}$
m-hydroxyphenylacetic acid	n.d.	n.d.	9.34 ± 1.74 * $^{+}$
o-hydroxyphenylacetic acid	n.d.	4.03 ± 0.79 *	26.12 ± 3.58 * $^{+}$
Hydroxyphenylacetic acid sulphate	n.d.	n.d.	n.d.
Hydroxyphenylacetic acid glucuronide	n.d.	3.32 ± 1.18 *	5.56 ± 1.27 *
Dihydroxyphenylacetic acid	n.d.	n.d.	n.d.
Dihydroxyphenylacetic acid sulphate	25.61 ± 4.22	58.34 ± 5.25 *	81.61 ± 13.02 *
Dihydroxyphenylacetic acid glucuronide	n.d.	8.27 ± 0.44 *	17.53 ± 5.02 *
Phenylpropionic acid	n.d.	8.13 ± 0.60 *	166.5 ± 53.88 *
Hydroxyphenylpropionic acid	n.d.	7.94 ± 3.28 *	89.37 ± 20.27 * $^{+}$
Hydroxyphenylpropionic acid sulphate	56.68 ± 32.77	65.41 ± 2.56 *	545.9 ± 64.26 * $^{+}$
Hydroxyphenylpropionic acid glucuronide	n.d.	n.d.	8.04 ± 2.12 * $^{+}$
Dihydroxyphenylpropionic acid	n.d.	n.d.	n.d.
Dihydroxyphenylpropionic acid sulphate	2.10 ± 0.12	1.74 ± 0.04 *	3.21 ± 0.16 * $^{+}$
Dihydroxyphenylpropionic acid glucuronide	n.d.	1.31 ± 0.05 *	3.58 ± 0.28 * $^{+}$
Vanillic acid	n.d.	4.69 ± 0.95 *	11.12 ± 2.71 * $^{+}$
Vanillic acid sulphate	n.d.	n.d.	1.84 ± 0.38 * $^{+}$
Homovanillic acid sulphate	0.97 ± 0.41	7.67 ± 0.48 *	36.73 ± 5.86 * $^{+}$
Hippuric acid	5.64 ± 0.33	13.02 ± 3.42 *	123.09 ± 10.52 * $^{+}$
Catechol sulphate	14.93 ± 1.14	297.7 ± 33.13 *	2247 ± 219.8 * $^{+}$
Catechol glucuronide	n.d.	n.d.	32.89 ± 5.22 * $^{+}$
Methyl catechol sulphate	n.d.	185.2 ± 15.34 *	1293 ± 104.4 * $^{+}$
Methyl catechol glucuronide	n.d.	4.40 ± 1.56 *	119.41 ± 12.37 * $^{+}$
p-hydroxybenzoic acid	0.45 ± 0.16	6.72 ± 0.96 *	26.48 ± 6.19 * $^{+}$
Hydroxybenzoic acid	n.d.	151.13 ± 31.43 *	1219 ± 179.6 * $^{+}$
Hydroxybenzoic acid sulphate	0.45 ± 0.05	2.13 ± 0.19 *	14.97 ± 1.40 * $^{+}$
Hydroxybenzoic acid glucuronide	n.d.	n.d.	4.08 ± 0.43 * $^{+}$

Supplementary Table 2: Kinetics of ferulic acid metabolites in human plasma.
Plasma pharmacokinetics of ferulic acid-derived phenolic compounds in 5 healthy volunteers 1, 3, 6 and 24 hours after consumption of 500 mg of ferulic acid were determined by UHPLC. Concentrations of the metabolites are expressed as the area under the curve (AUC, µmol/L min^{-1}) and peak concentration (C_{max}, µM) are mean±SEM of 5 subjects. Lower panel: Representation of the time-kinetics of absorption of the five major metabolites detected in plasma. *($p<0.05$) *vs* C57BL/6J or $^{+}$($p<0.05$) *vs* ApoE-/- HFD. n.d. indicates non-detected compounds.

Phenolic metabolite	AUC total (µmol/L min^{-1})	C_{max} (µM)
Ferulic acid	2406.2 ± 729.0	16.25 ± 7.68
Isoferulic acid	409.3 ± 139.7	2.95 ± 1.50
Ferulic acid sulphate	13412 ± 4665	39.79 ± 8.12
Ferulic acid glucuronide	3908.4 ± 963.3	9.18 ± 1.52
p-coumaric acid	n.d.	n.d.
Coumaric acid sulphate	n.d.	n.d.
Coumaric acid glucuronide	n.d.	n.d.
Caffeic acid	589.1 ± 87.91	2.12 ± 0.46
Caffeic acid sulphate	74.76 ± 67.36	0.08 ± 0.07
Hydroxyphenylacetic acid	n.d.	n.d.
Hydroxyphenylacetic acid sulphate	11619 ± 9568	7.15 ± 5.81
Hydroxyphenylacetic acid glucuronide	74.74 ± 22.11	0.18 ± 0.04
Dihydroxyphenylacetic acid	4362.2 ± 1280.0	3.98 ± 1.30
Methoxybenzoic acid	84.84 ± 34.89	0.45 ± 0.21
Vanillic acid	589.1 ± 87.91	2.12 ± 0.46
Vanillic acid sulphate	304.6 ± 69.11	0.51 ± 0.10
Vanillic acid glucuronide	241.0 ± 51.53	0.30 ± 0.07
Protocatechuic acid	257.7 ± 136.0	0.25 ± 0.12
Homovanillic acid glucuronide	n.d.	n.d.
p-hydroxybenzoic acid	1417.6 ± 591.4	1.40 ± 0.58
Hydroxybenzoic acid sulphate	237.72 ± 160.0	0.25 ± 0.17
Dihydroferulic acid	1243.3 ± 577.3	1.92 ± 0.53
Dihydroferulic acid sulphate	746.3 ± 336.4	0.89 ± 0.22
Dihydroferulic acid glucuronide	126.7 ± 64.37	0.15 ± 0.07
Phenylacetic acid	n.d.	n.d.
Hydroxyphenylpropionic acid	2238.2 ± 514.1	2.54 ± 0.59
Hydroxyphenylpropionic acid sulphate	617.9 ± 96.97	0.73 ± 0.10
Catechol sulphate	n.d.	n.d.
Methyl catechol sulphate	n.d.	n.d.
Methyl catechol glucuronide	n.d.	n.d.
Hydroxybenzoic acid	n.d.	n.d.
Hippuric acid	n.d.	n.d.



Supplementary Table 3: Serum values of lipids, glucose and liver enzymes in the human treatment study.

Serum lipids, glucose and liver enzymes were determined at baseline (control condition) and after 15 days of ferulic acid consumption in serum. LDL-C: low-density lipoprotein cholesterol. HDL-C: high-density lipoprotein cholesterol. TG: triglycerides. AST: aspartate aminotransferase. ALT: alanine aminotransferase. GGT: gamma-glutamyl transferase. CRP: C-reactive protein. Results are expressed as mean ± SEM of 5 subjects.

	Control	Ferulic acid	p-value
Total cholesterol	194.6 ± 6.01	190.4 ± 2.98	0.203
LDL-C	114.4 ± 4.74	109.0 ± 7.39	0.411
HDL-C	66.2 ± 8.00	66.8 ± 7.72	0.958
TG	91.8 ± 13.2	93.8 ± 18.82	0.510
Glucose	99.6 ± 5.22	85.6 ± 6.12	0.766
AST	21.8 ± 0.97	22.2 ± 1.46	0.445
ALT	21.0 ± 4.88	24.8 ± 6.0	0.700
GGT	22.6 ± 8.21	29.6 ± 14.5	0.300
Bilirubin	0.6 ± 0.15	0.54 ± 0.07	0.190
CRP	0.54 ± 0.19	0.44 ± 0.09	0.151

Supplementary Table 4: Primer sequences and conditions used for real-time PCR.

Gene name (Mmu)	Abbreviation	Forward primer (5' - 3')	Reverse primer (5' - 3')	Annealing temperature	PCR product (bp)	Accession
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	Pgc-1a	CAGACCTGACACAACGCGGACAG	CCTGCGCAAGCTTCTCTGAGC	62	343	NM_008904.2
Peroxisome proliferator-activated receptor gamma coactivator 1-beta	Pgc-1β	CTCCAGGCAGGTTCAACCC	GGGCCAGAAGTTCCCTTAGG	62	83	NM_133249.2
Nuclear respiratory factor-1	Nrf-1	GCATTGAGCTACTGACAGAC	CTGTGTCCTGGATCTTCCTT	57	117	NM_001164226.1
Nuclear respiratory factor-2	Nrf-2	TCAGTGACTCGGAAATGGAG	TTCACGCATAGGAGCACTGT	57	143	U20532
Transcription factor A-mitochondrial	Tfam	AGTCAGCTGATGGGTATGGAGAA	TGCTGAACGAGGTCTTTTTGG	59	114	NM_009360.4
Mitofusin-1	Mfn1	TTGCCACAAGCTGTGTTCGG	TCTAGGGACCTGAAAGATGGGC	61	148	NM_024200.4
Mitofusin-2	Mfn2	AGAGGCAGTTTGAGGAGTGC	ATGATGAGACGAACGGCCTC	61	103	NM_001285920.1
Optic Atrophy 1	Opa1	TCTGAGGCCCTTCTCTTGTT	TCTGACACCTTCCTGTAATGCT	57	98	NM_001199177.1
Mitochondrial fission 1	Fis1	ACGAAGCTGCAAGGAATTTTGA	AACCAGGCACCAGGCATATT	57	98	NM_001163243.1
Beclin-1	Beclin-1	CTCCATTACTTACCACAGCCCA	AAATGGCTCCTCTCCTGAGT	59	77	NM_019584.3
18S ribosomal RNA	18S	TTGATTAAGTCCCTGCCCTTTGT	CGATCCGAGGGCCTCACTA	60	77	NR_046237.1

Gene name (hs)	Abbreviation	Forward primer (5' - 3')	Reverse primer (5' - 3')	Annealing temperature	PCR product (bp)	Accession
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PGC-1a	TGCCCTGGATTGTTGACATGA	TTTGTCAGGCTGGGGGTAGG	60	117	NM_013261.3
Mitofusin-1	MFN1	GAGAAAATACAAAACAATTCAAAGCTC	GCTTGAAGGTAGAAACTGCTTAGTAAA	59	96	NM_033540.2
Mitochondrial fission 1	FIS1	GAAAGATGGACTCGTGGGCA	GAACAGGGAAAGGACAGCGA	59	172	NM_016068.2
18S ribosomal RNA	18S	TTGATTAAGTCCCTGCCCTTTGT	CGATCCGAGGGCCTCACTA	60	77	NR_046237.1

RESUMEN DE RESULTADOS

Resumen de resultados en ratones deficientes en apolipoproteína E (ApoE-/-)

Hipercolesterolemia

Suero:
- Reducción del colesterol total (CT) y del ratio CT/HDL-C (HFD 1% y HFD 5%)
- Incremento del colesterol HDL (HFD 1% y HFD 5%)

Válvula aórtica:
- Reducción de la placa aterosclerótica (LFD 5% y HFD 5%)
- Menor deposición de lípidos en la placa (HFD 1% y HFD 5%)

Aorta:
- Reducción de la placa aterosclerótica (HFD 5%)

Hígado:
- Menor actividad de HMG-CoA reductasa (HFD 1% y HFD 5%)
- Menor esteatosis (LFD 1%, HFD 1% y HFD 5%)
- Mayor expresión de PPAR-α (HFD 5%)

Heces:
- Mayor excreción fecal de colesterol (HFD 5%)

Estrés oxidativo

Aorta:
- Menor producción de anión superóxido (HFD 1%)
- Menor expresión de Nox-1 y $p47^{phox}$ (HFD 1% y HFD 5%)
- Menor expresión de $p22^{phox}$ (HFD 1%)
- Mayor expresión de CuZn-SOD (HFD 1%)
- Regulación de la expresión de Mn-SOD (HFD 1% y HFD 5%)
- Menor acúmulo de oxLDL (HFD 1% y HFD 5%)
- Incremento del ratio GSH/GSSG (HFD 5%)
- Reducción de la expresión de peroxiredoxina SO_3 y de la peroxidación lipídica (MDA) (HFD 5%)

Suero:
- Reducción los metabolitos de NO (HFD 1% y HFD 5%)

Mesentérica:
- Menor producción de anión superóxido (HFD 1%)
- Menor expresión de Nox-1 (HFD 1%)

ApoE-/-

LFD: 14% kcal grasa
LFD 1%: LFD + 1% (p/p) EESA
LFD 5%: LFD + 5% (p/p) EESA
HFD: 42% kcal grasa + 0.15% (p/p) colesterol
HFD 1%: HFD + 1% (p/p) EESA
HFD 5%: HFD + 5% (p/p) EESA

Inflamación

Válvula aortica:
- Menor infiltración de macrófagos (HFD 1% y HFD 5%)

Aorta:
- Menor expresión de VCAM-1, ICAM-1 e iNOS (HFD 1% y HFD 5%)
- Menor expresión de TNF-α soluble y COX-2 (HFD 5%)
- Menor activación de NF-κβ (HFD 1% y HFD 5%)
- Menor producción de anión superóxido (HFD 1%)

Suero:
- Reducción de los niveles de AST (HFD 1% y HFD 5%)

Apoptosis y senescencia

Aorta:
- Menor acortamiento de telómeros (HFD 5%)
- Menor apoptosis de células endoteliales (HFD 5%)
- Menor expresión de p53, p16, Bax y del ratio Bcl-2/Bax (HFD 5%)

MNC esplénicas
- Menor acortamiento de telómeros (HFD 5%)
- Menor actividad telomerasa (HFD 5%)
- Mayor expresión de TRF2, p53, Bcl-2 y Bax (HFD 5%)

Disfunción vascular

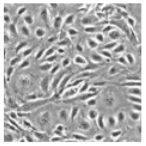
Aorta:
- La suplementación de la dieta con EESA no revirtió la disfunción endotelial inducida por HFD en animales de 28 semanas, pero si en los de 10 semanas de edad (HFD 1% y HFD 5%)

Mesentérica:
- Mejora en la función endotelial (HFD 1% y HFD 5%)
- Incremento en la liberación de NO (HFD 1% y HFD 5%)
- Reducción de la inhibición de eNOS por fosforilación en Thr495 (HFD 5%)
- Menor deposición de colágeno (HFD 5%)
- Normalización de la rigidez (β value) (LFD 1%, LFD 5% y HFD 5%)
- Mejora de las alteraciones estructurales y mecánicas del vaso (LFD 1%, LFD 5%, HFD 1% y HFD 5%)

Disfunción mitocondrial

Aorta:
- Recuperación de la expresión de ARNm de genes implicados en el ciclo mitocondrial: *Pgc-1a, Pgc-1β, Nrf-1, Mfn1, Mnf2, Fis1, Beclin-1* (HFD 5%)
- Incremento en la expresión de AMPK y de su activación mediante fosforilación (HFD 5%)
- Menor acetilación de PGC-1α (HFD 5%)

Resumen de resultados de ensayos *in vitro* y en humanos



BAEC

BAEC inducidas con H_2O_2 en presencia de EESA (367.6 mg/L), ácido ferúlico (5.5 µM), γ-oryzanol (5.5 µM), β-sitosterol (1.5 µM) o γ-tocotrienol (0.12 µM)

EESA:

- Incremento del ratio GSH/GSSG
- Reducción de la peroxidación lipídica
- Incremento en la fosforilación de AMPK

Ácido ferúlico:

- Incremento del ratio GSH/GSSG
- Reducción de la peroxidación lipídica
- Incremento en la fosforilación de AMPK

γ-oryzanol:

- Incremento del ratio GSH/GSSG

γ-tocotrienol:

- Reducción de la peroxidación lipídica

PBMC EPC

PBMC y EPC aisladas de humanos tratados durante 15 días con 500 mg de ácido ferúlico al día

PBMC:

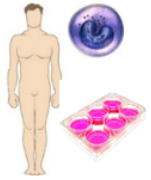
- Reducción de la actividad de NADPH oxidasa basal y tras inducción con PMA

- Reducción de la apoptosis y necrosis inducida por H_2O_2

- Incremento en la expresión de ARNm de los marcadores del ciclo mitocondrial PGC-1a y MFN1

EPC:

- Incremento de la diferenciación y proliferación de EPC



PBMC

PBMC aisladas de humanos y tratadas *in vitro* con EESA (20 µg/ml), ácido ferúlico (20 µg/ml o 5.5 µM), o γ-oryzanol (20 µg/ml)

EESA, ácido ferúlico y γ-oryzanol (20 µg/ml):

- Reducción del perfil proinflamatorio de monocitos inducidos con LPS, disminuyendo la subpoblación de monocitos intermedios (CD14++CD16+) e incrementando la de monocitos no clásicos (CD14-CD16++)

- Reducción de la expresión de ARNm de marcadores de macrófagos M1 (CD64, CD80), IL-6 y TNF-α e incremento de marcadores M2 (MR, CD200r)

Ácido ferúlico (5.5 µM):

- Reducción de la producción de anión superóxido inducida por H_2O_2

- Reducción de la apoptosis inducida por H_2O_2

Rata Wistar

Ratas Wistar sondadas con 10 g/kg. Inducción de anillos de aorta con endotelina 1 en presencia del suero de las ratas sondadas o de EESA (367.6 mg/L), ácido ferúlico (5.5 µM), o γ-oryzanol (5.5 µM)

Suero, orina y heces:

- Determinación de la biodisponibilidad de los compuestos fenólicos presentes en el EESA.

Aorta:

- Reducción de la producción de anión superóxido inducida por endotelina 1 en presencia de EESA, ácido ferúlico o del suero de las ratas sondadas con EESA

DISCUSIÓN

El presente trabajo recoge los efectos de un extracto enzimático de salvado arroz (EESA) sobre el desarrollo de la aterosclerosis y aquellas alteraciones funcionales que pueden desarrollarse como consecuencia de la misma. Mediante el uso de un modelo clásico para el estudio de la aterosclerosis, los ratones deficientes en apolipoproteína E (ApoE-/-), se ha ensayado el efecto de la suplementación con EESA de dietas con un contenido bajo (LFD) o elevado en grasas y colesterol (HFD) sobre la hiperlipidemia y el metabolismo de colesterol. Se ha prestado especial atención al estudio del estrés oxidativo y el estado inflamatorio a nivel vascular como hilos conductores del desarrollo de aterosclerosis y de otros procesos asociadas a ella: disfunción y remodelado vascular, esteatosis, apoptosis, senescencia celular y disfunción mitocondrial. Finalmente, en el EESA coexisten numerosas moléculas bioactivas que pueden metabolizarse y dar lugar a otros compuestos también responsables de las actividades observadas. En un intento de acercarnos a la compresión de la biodisponibilidad de los principios activos del EESA se han medido los niveles de ácido ferúlico y sus metabolitos en plasma, orina y heces a diferentes tiempos tras la administración oral de EESA. Adicionalmente, hemos realizado ensayos *in vitro* con los compuestos mayoritarios a las concentraciones biodisponibles para determinar su actividad.

1. ***Efectos del EESA sobre el colesterol plasmático y mecanismos de la acción hipocolesterolemiante en ratones ApoE-/-***

Para que se inicie el proceso aterosclerótico, es necesario un primer daño en el endotelio que desencadene una respuesta inflamatoria y aumento de la permeabilidad vascular a lípidos y células del sistema inmune. A continuación, la oxidación del colesterol en la pared arterial y su fagocitosis por parte de macrófagos inician el conjunto de eventos que subyacen a la enfermedad. Aunque parece necesaria la presencia de elevados niveles de colesterol para que se inicie el proceso aterosclerótico, ya en 1819 el irlandés Samuel Black apuntó la aparente incoherencia de que los franceses, a pesar de tener un consumo elevado en grasas saturadas, presentaban una prevalencia sorprendentemente baja de infartos de miocardio. Pero no fue hasta los años 80 cuando la antigua creencia de "sin colesterol no hay lesión" se precisó con el descubrimiento de la **paradoja francesa**, creando un nuevo paradigma en la comprensión de los mecanismos etiológicos de la enfermedad. En el año 1987 se asoció la menor prevalencia de eventos cardiovasculares de los franceses al elevado consumo de vino, rico en antioxidantes y a un estilo de vida activo (Richard et al., 1987). Este hecho originó la **reformulación de la teoría de "la respuesta al daño"** que postulaba que la respuesta inflamatoria y las lesiones proliferativas que tienen lugar en la pared arterial son ocasionadas por la hipercolesterolemia. Las nuevas teorías

defienden que si bien el daño endotelial es necesario, las causas del mismo no se limitan a un elevado colesterol plasmático, sino que la activación endotelial puede darse por múltiples casusas, como un estado de estrés oxidativo elevado, el tabaquismo, la hipertensión, las consecuencias metabólicas de la diabetes o el acumulo de colesterol oxidado en la pared (Ross, 1986; Ravnskov, 2003).

Dada la importancia que el elevado contenido en colesterol plasmático tiene para el desarrollo de la aterosclerosis, se investigaron los efectos de la suplementación con EESA sobre el **perfil lipídico** y sus **mecanismos hipolipemiantes** en las dietas LFD y HFD. Se observó una reducción significativa del colesterol total y un incremento del colesterol HDL en los grupos HFD 1% y 5%, que llevó a la reducción del ratio colesterol total/colesterol HDL. Asimismo, el grupo HFD 5% también presentó una bajada en los niveles de triglicéridos. Sin embargo, ninguna de estas mejoras se presentó en los animales alimentados con la dieta LFD, la cual no contiene colesterol. Esto nos lleva a pensar que la presencia de colesterol dietético puede ser un factor clave para los mecanismos protectores mediados por el EESA.

Existe un gran consenso acerca de los efectos hipolipemiantes de los **fitosteroles**, que actúan de forma dosis dependiente bloqueando la absorción de colesterol a nivel intestinal por varios mecanismos (Ikeda et al., 1988; Trautwein et al., 2003; Plat et al., 2005). Los fitosteroles están presentes en el EESA en forma libre y en gran medida como parte de la molécula de **γ-oryzanol**. Los efectos del EESA sobre la excreción de colesterol fueron investigados en las heces de los ratones ApoE-/-, encontrando una mayor excreción de colesterol en los grupos alimentados con una dieta HFD, que se potenció cuando los animales fueron tratados con EESA al 5%. Sin embargo, los ratones alimentados con la dieta LFD suplementada con EESA no modificaron su excreción fecal de colesterol. La menor excreción de colesterol en el grupo LFD así como la falta de efecto del tratamiento puede explicarse por la ausencia de colesterol en esta dieta, que se traduce en un menor contenido de colesterol intestinal. Así, la excreción fecal de colesterol de este grupo se correspondería con la excreción basal de colesterol de procedencia biliar, sin que el EESA ejerza ningún efecto reseñable sobre los eficaces mecanismos fisiológicos de reciclaje de colesterol. Otra posible explicación es que en un estado de mayor hipercolesterolemia inducida por el aporte suplementario de colesterol en la dieta HFD, el γ-oryzanol presente en el EESA induzca una mayor síntesis de ácidos biliares y potencie el flujo hacia el intestino, incrementando su eliminación con las heces, como han sugerido otros autores (Seetharamaiah et al., 1990). Del mismo modo, el hecho de que sólo el en grupo HFD 5% EESA se obtuviera un efecto potenciador sobre la excreción fecal de colesterol se explica por el mayor aporte en fitosteroles de esta dieta frente a la del 1%.

El γ-oryzanol es también fuente de **ácido ferúlico**, que tras ser absorbido alcanza el hígado e inhibe allí a la enzima HMG-CoA-Reductasa (Wang et al., 2015). Esta inhibición fue observada en los ratones alimentados con la dieta HFD independientemente de la dosis, pero no en la dieta LFD. Otros autores han descrito previamente mejoras en el perfil lipídico de ratas Wistar alimentadas con una dieta alta en grasas y colesterol tras ser suplementada con un extracto enzimático de salvado de arroz de similares características al empleado en este trabajo, y han atribuido el efecto al incremento de la excreción fecal de colesterol y a la inhibición de la actividad de HMG-CoA-Reductasa (Wang et al., 2014). A diferencia de lo presentado en este trabajo, los ratones ApoE-/- no presentaron una menor actividad de HMG-CoA-Reductasa compensatoria al aporte de colesterol de la dieta. Este hecho podría explicarse por diferencias inter-especie o por la alteración del metabolismo del colesterol que supone la delección de la apolipoproteína E (Moghadasian et al., 2001).

2. *Efectos del EESA sobre la aterogénesis en ratones ApoE-/-*

Los animales alimentados con una dieta HFD suplementada con EESA presentaron un menor desarrollo de aterosclerosis en la válvula aórtica y cayado. La menor cantidad de lípidos aunado a una menor presencia de ROS explican la menor presencia de partículas de oxLDL en la aorta de los ratones alimentados con la dieta HFD tanto al 1 como al 5% de EESA. Además, la reducción del estado inflamatorio, de la expresión de las moléculas de adhesión VCAM-1 e ICAM-1 y especialmente de la consecuente infiltración de macrófagos en la placa, explican el menor desarrollo de la placa y del depósito de lípidos en la misma. Por el contrario, los grupos alimentados con la dieta LFD no se vieron beneficiados de las mejoras en el perfil lipídico, de la bajada en la expresión de moléculas de adhesión ni de la reducción de la infiltración de macrófagos en la placa, lo que podría estar relacionado con la ausencia de colesterol en esta dieta, como se ha comentado previamente. Sin embargo, el grupo al que se le suplementó la dieta LFD con un 5% de EESA mostró una reducción del desarrollo de la placa en la válvula aortica. Este hecho debe atribuirse a otros efectos antiinflamatorios o antioxidantes no evaluados en este trabajo.

3. *Efectos del EESA sobre el estrés oxidativo en ratones ApoE-/-*

El estrés oxidativo juega un papel crucial en el desarrollo de la aterosclerosis. La mitocondria es la principal fuente de radical superóxido ($O_2^{\cdot-}$) a nivel celular. El radical superóxido es el radical más abundante y a partir del cual pueden generarse otras ROS cada vez más reactivas que propagarán el daño oxidativo **(Figura 22).** El **radical superóxido**

también puede formarse como producto de enzimas como la NADPHox que se inducen típicamente en estados inflamatorios como la aterosclerosis. Otra enzima que produce ROS en abundancia es la óxido nítrico sintasa inducible (iNOS), fuente de **NO**. Adicionalmente, los radicales libres también pueden generarse como consecuencia de factores ambientales como la contaminación, la dieta o el tabaco. Por ello es necesario que las células dispongan de mecanismos efectivos para neutralizar estas especies. Estos mecanismos pueden clasificarse en enzimáticos (superóxido dismutasa (SOD), catalasa, GPx, entre otros) y no enzimáticos como el glutatión.

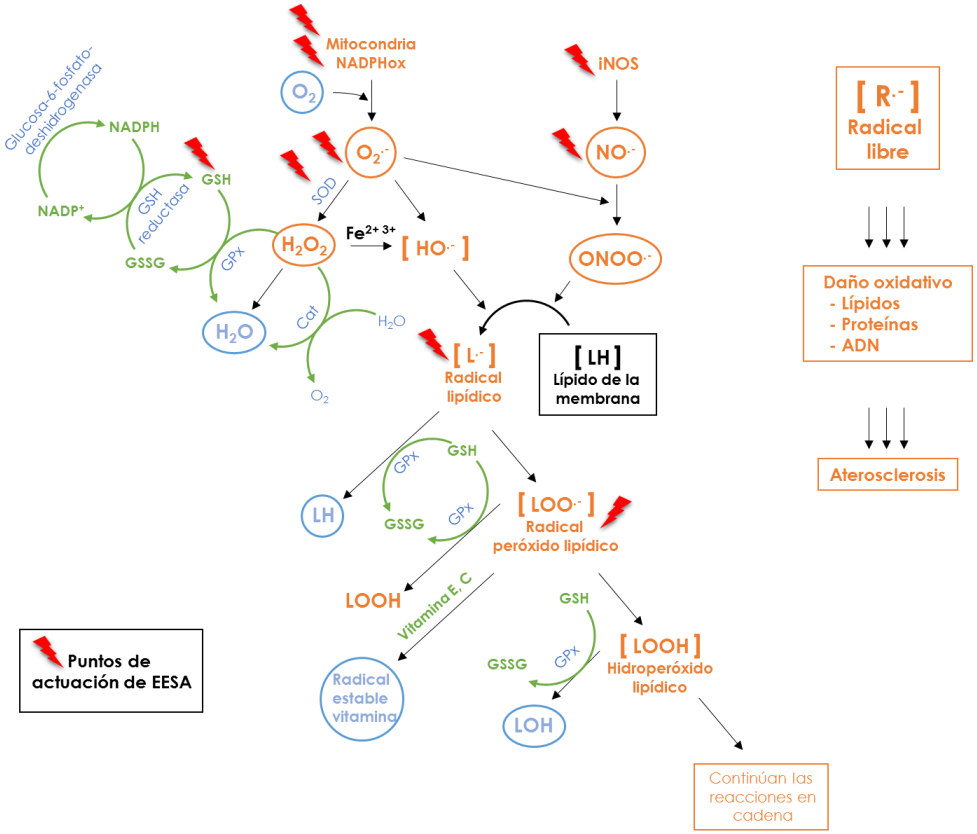


Figura 22: Fuentes y reacción en cadena de las especies reactivas de oxígeno (ROS).

En nuestro trabajo, los ratones ApoE-/- presentaron una mayor cantidad de anión superóxido en la aorta y arterias mesentéricas y de metabolitos del NO en suero. Los efectos inhibitorios sobre la actividad enzima **NADPHox** estudiados *in vitro* fueron confirmados en los tratamientos de ratones ApoE-/-. Los animales cuya dieta HFD fue suplementada con un 1% de EESA presentaron una menor producción de anión superóxido relacionada con la menor expresión de NADPHox, aunque la regulación del enzima fue diferente según el tipo

arterial. En las arterias mesentéricas se observó una menor expresión de la subunidad Nox-1 mientras que la expresión de $p22^{phox}$ permaneció sin cambios. Por el contrario las tres subunidades evaluadas (Nox-1, $p22^{phox}$ y $p47^{phox}$) redujeron su expresión en la aorta con la dosis del 1%. A diferencia de lo ocurrido en el lecho mesentérico donde no se produjo ningún cambio, la dosis del 5% redujo la expresión de Nox-1 y $p47^{phox}$ en la aorta, aunque no la de $p22^{phox}$. La menor producción de anión superóxido en la aorta de los animales HFD 1% estaría también justificada por la mayor expresión de la enzima antioxidante citoplasmática CuZn-SOD, que rápidamente neutraliza al anión superóxido en H_2O_2. A diferencia del anión superoxido, los metabolitos del NO se redujeron en la misma amplitud en ambos grupos de tratamiento, como consecuencia de la inhibición paralela de la expresión de la enzima iNOS.

Resulta llamativo que si bien los mecanismos antioxidantes que tienen un efecto a corto plazo parecen carecer de efecto en el grupo de tratamiento del 5% EESA (CuZn-SOD, actividad de aconitasa, anión superóxido, NADPHox), los productos más tardíos de la oxidación (GSH/GSSG, oxidación de peroxiredoxina, peroxidación lipídica y acúmulo de LDL oxidadas en la aorta) mejoran ampliamente en el grupo del 5% EESA. La menor protección a corto plazo de la dosis del 5% puede estar relacionada con un mayor aporte de tocotrienoles que una vez biotransformados en tocoferoles interfieran en la actividad antioxidante de los primeros (Bowry et al., 1993; Khor et al., 1995), mientras que las mejoras a largo plazo podrían deberse a la reducción del estrés oxidativo derivado de la bajada de los lípidos plasmáticos, más potente en la dosis del 5%.

4. Capacidad antinflamatoria del EESA en ratones ApoE-/-

Durante el proceso inflamatorio, los macrófagos activados liberan una gran cantidad de ROS que pueden oxidar lípidos, proteínas y ácidos nucleicos, resultando en un cambio de sus estructuras y función. Estrés oxidativo e inflamación están por lo tanto íntimamente interconectados y pueden potenciarse mutuamente a través de un mecanismo mediado por NF-κβ (Biswas, 2016) **(Figura 23).** Se conoce que el TNF-α es uno de los principales activadores de NF-κβ a través de la fosforilación de Ικκ.

Figura 23: Interrelación entre estrés oxidativo e inflamación a través de las especies reactivas de oxígeno (ROS).

Niveles elevados de citoquinas proinflamatorias como **TNF-α e IL-6** aparecen como resultado de la activación de macrófagos M1 en el área de la placa de ateroma y favorecen su desarrollo. Por el contrario, macrófagos M2 liberarán **e**, que tiene efectos antiinflamatorios. Los niveles plasmáticos de estas tres citoquinas fueron medidos en el suero de los ratones ApoE-/-, sin encontrar diferencias significativas en los ratones tratados con EESA, aunque si una mayor expresión en la dieta HFD en comparación con LFD. Esta ausencia de efecto del EESAA puede explicarse por el hecho de que si bien la producción local de citoquinas en la placa de ateroma y la posible atenuación por parte del EESA podrían detectarse en el microambiente del vaso, una vez liberadas a sangre el efecto se pierde pues la aterosclerosis se trata de un proceso inflamatorio crónico de bajo grado a nivel sistémico en el que no se encuentran grandes cantidades de citoquinas, como sería el caso de un proceso inflamatorio agudo. Esta hipótesis viene reforzada por la menor expresión de TNF-α en el tejido aórtico de los animales tratados con EESA. Los animales tratados con EESA presentaron menor cantidad de la subunidad p65 de NF-κβ en el núcleo, lo que se tradujo en una menor expresión de las enzimas proinflamatorias COX-2 e iNOS en el tejido aórtico. La menor expresión de iNOS condujo a la reducción concentración de los metabolitos de NO en el suero de los animales tratados con EESA.

Los efectos antiinflamatorios fueron levemente superiores en la dosis del 5% EESA frente a la del 1%, que podría explicarse por la mayor expresión hepática de PPAR-α, relacionada con la regulación del metabolismo lipídico hepático y con la modulación de

reacciones antiinflamatorias mediadas por lípidos, a través de la inhibición de NF-κβ (Varga et al., 2011).

En la línea de estos efectos antiinflamatorios, estudios previos de nuestro grupo demostraron el potencial antiinflamatorio de la suplementación con EESA en la vasculatura y tejido adiposo de ratas Zucker (Justo et al., 2013a, Candiracci et al., 2014; Justo et al., 2014) y en el modelo de obesidad inducida por la dieta (DIO) (Justo et al., 2016) mediante la disminución de la liberación de TNF-α y de la expresión de iNOS y COX-2. Además, otros autores han demostrado los efectos antiinflamatorios del ácido ferúlico y γ-oryzanol mediado por la inhibición de la activación de NF-κβ y de la liberación de citoquinas proinflamatorias (Akihisa et al. 2000; Islam et al., 2011; Sakai et al., 2012). Además se ha descrito que los efectos hipolipemiantes del γ-oryzanol y los fitosteroles repercuten positivamente sobre la inflamación (Islam et al., 2011; Tall et al., 2015).

5. ***Actividad del EESA sobre otras alteraciones asociadas con el proceso aterosclerótico en ratones ApoE-/-***

La reducción del desarrollo de la placa aterosclerótica puede ser a la vez causa y consecuencia de otras alteraciones asociadas al desarrollo de patologías cardiovasculares y que serán discutidas a continuación.

a) Disfunción endotelial

La disfunción endotelial puede ser detectada antes de que ocurran los cambios estructurales característicos de la pared arterial, por lo que es considerada como un marcador temprano de para el pronóstico de aterosclerosis. Además, muchos de los factores de riesgo para la aterosclerosis, como la diabetes, el tabaquismo la hipercolesterolemia o la hipertensión, también causan disfunción endotelial (Davignon et al., 2004). La hipercolesterolemia promueve la interacción inhibitoria de caveolina con la enzima eNOS, reduciendo así su producción de **NO** (Feron et al., 1999). El NO, además de mediar la vasodilatación dependiente de endotelio, tiene un papel muy importante en el desarrollo de la aterosclerosis ya que tiene propiedades antiinflamatorias (inhibe la adhesión leucocitaria), antioxidantes y antitrombóticas (limita la adhesión y agregación plaquetaria y la expresión de PAI-1) (Napoli et al., 2006). Además, el NO reduce la permeabilidad endotelial y el tono vascular, reduciendo el flujo de lipoproteínas hacia la pared (Cardona-Sanclemente et al., 1995). Sin embargo, la producción excesiva de NO derivada de la isoforma inducible por procesos inflamatorios **iNOS**, puede ocasionar daños

en la pared vascular, especialmente cuando se combina con otras especies reactivas de oxígeno. La gran presencia de ROS, característica de la aterosclerosis, reducen la biodisponibilidad de NO para la vasodilatación **(Figura 22)**. Además, una característica distintiva de las células endoteliales expuestas a la hipercolesterolemia es la reducida capacidad para liberar **EDHF** (Henry et al., 1995), lo que ocasiona un aumento del tono vascular. En este trabajo se ha evaluado la función endotelial de arterias de conductancia (aorta) y de resistencia (arterias mesentéricas). Si bien la función de la aorta es la de asegurar la distribución de sangre rica en oxígeno y nutrientes a todos los tejidos, las arterias mesentéricas juegan un papel crucial en el control del flujo sanguíneo y presión arterial, lo cual apunta a un estrecho control recíproco entre ambos lechos vasculares. Además, las arterias de resistencia, a diferencia de las arterias de conductancia, no desarrollan placas de ateroma, pero pueden sufrir disfunción y remodelado de la estructura de su pared al igual que la aorta.

En el modelo de ratones ApoE-/-, se observó que la vasodilatación dependiente de endotelio esta mediada por el NO en las **arterias mesentéricas**, a diferencia de los ratones de genotipo salvaje, en los que el EDHF tuvo una mayor participación. Este hecho fue corroborado por una menor expresión de los canales de potasio IK_{Ca} y SK_{Ca} en los ratones ApoE-/-. Además, las arterias mesentéricas de los ratones ApoE-/- presentaron una menor vasodilatación dependiente de endotelio en respuesta a acetilcolina independientemente del contenido graso de la dieta. Si bien el tratamiento con EESA no produjo ningún cambio en la dieta LFD, la respuesta a acetilcolina se mejoró notablemente en la dieta HFD. Esta mejora en la vasodilatación se relacionó con una mayor liberación de NO en los grupos tratados con EESA, derivada de la menor fosforilación inhibitoria en el residuo Thr495 de la enzima eNOS. Además, se observó que la HFD ejerce un efecto deletéreo sobre la expresión de eNOS, sin que afecte al nivel de fosforilación en el residuo Ser1177, asociado a la activación del enzima. En la línea de estas observaciones, otros autores han descrito los efectos perjudiciales de una dieta alta en grasa sobre la vasodilatación de arterias de resistencia (Heinonen et al., 2014), y en estudios previos de nuestro grupo se demostraron los efectos beneficiosos de la suplementación de la dieta con EESA sobre la vasodilatación mediada por NO de microvasos de ratas Zucker obesas (Favero et al., 2014).

Por otra parte, la evaluación de la funcionalidad vascular en la **aorta** también reveló una disfunción endotelial, que fue mayor en los animales alimentados con la dieta HFD en comparación con la dieta LFD. En la aterosclerosis, además de la disfunción endotelial generalizada derivada del estado de hipercolesterolemia, se produce una

disfunción endotelial de mayor grado focalizada en los lugares donde se están desarrollando las placas de ateroma. El tratamiento con EESA consiguió reducir el desarrollo de la placa en ambas dietas, así como el estrés oxidativo en la dieta HFD, lo que podría mejorar la disponibilidad de NO. Sin embargo, la función endotelial no se vio beneficiada de estas mejoras en ninguna de las dietas cuando los ratones tenían una edad de 28 semanas en el momento del sacrificio. Para comprobar si los efectos beneficiosos del EESA pudieran estar siendo enmascarados por un desarrollo excesivo de la placa o un nivel de estrés oxidativo que el EESA no pudiera compensar de manera suficientemente eficiente para observar cambios en la función endotelial, se repitieron los experimentos, reduciendo la edad de sacrificio a 10 semanas. En este caso, los ratones tratados con EESA mostraron una mejor relajación dependiente de endotelio, independientemente de la dosis del extracto empleada, confirmando que la presencia de la placa, ausente en la aorta de los animales de 10 semanas, es un factor decisivo para apreciar los efectos de EESA sobre la función endotelial. Trabajos previos del grupo en aorta de ratas Zucker obesas y de otros autores en aorta de ratas hipertensas, encontraron mejoras en la función endotelial debidas a un menor estrés oxidativo, estado inflamatorio, a la reducción de la expresión de iNOS y al incremento en la expresión de eNOS (Justo et al., 2013a; Boonla et al., 2015).

Por otra parte, las **células progenitoras endoteliales (EPC)** juegan un papel muy importante para compensar la elevada tasa de apoptosis de células endoteliales que tiene lugar en la aterosclerosis. Su correcta funcionalidad se considera un marcador de salud vascular y podría contribuir a preservar la respuesta endotelial en condiciones de daños por estrés oxidativo o inflamatorio típicos de la aterosclerosis (Werner et al., 2005). En este sentido, el tratamiento con 500 mg de ácido ferúlico durante 15 días supuso una mejora en la proliferación de EPC y en su capacidad de migración, lo cual podría suponer un nuevo mecanismo del EESA, fuente de ácido ferúlico, para preservar la vasodilatación dependiente de endotelio, como se ha propuesto para otros alimentos y bebidas ricos en polifenoles (Zhu et al., 2004; Balestrieri et al., 2008; Felice et al., 2012; Lucchesi et al., 2014; Choi et al., 2015).

b) <u>Remodelado vascular</u>

El desarrollo de placas de aterosclerosis da lugar a cambios adaptativos de las propiedades estructurales y miogénicas de las arterias para asegurar el flujo de sangre y la estabilidad de la placa. La hipertrofia de vasos de conductancia y estabilización de la placa de ateroma han sido estudiadas en gran extensión, y no han formado parte de los objetivos de este trabajo. Sin embargo las **propiedades estructurales, miogénicas y**

mecánicas de los vasos de resistencia resultaron de interés para los objetivos planteados en este trabajo. En el lecho mesentérico, la menor rigidez de la pared permite una mayor distensibilidad, constituyendo mecanismos compensatorios para evitar el estrechamiento del lumen por un incremento del grosor de la pared.

En nuestro trabajo, los ratones ApoE-/- alimentados con la dieta HFD se caracterizaron por un incremento en la superficie transversal del vaso (CSA). Estos vasos, en lugar de presentar un lumen reducido, crecieron hacia el exterior, aumentando el diámetro del lumen de forma paralela a su CSA. Sin embargo, esta hipertrofia no consiguió compensar el estrés vascular (σ), característico de un estado avanzado de remodelado vascular, el cual, acompañado de la mayor rigidez que presentaron estos vasos es un marcador predictivo de enfermedad cardiovascular (Cecelja et al., 2012). La mayor rigidez podría estar relacionada con el mayor contenido en colágeno de las arterias del grupo HFD. Por el contrario, los animales que consumieron la dieta LFD mostraron un estadio inferior de remodelado vascular, pues a pesar de tener un CSA incrementado, consiguieron mantener un estrés vascular similar al grupo control. De acuerdo con nuestras observaciones, otros autores han encontrado mayores niveles de rigidez vascular acompañados de una mayor distensibilidad en microvasos de modelos de obesidad, isquemia, hipertensión hepática y aterosclerosis (Resch et al., 2009; Caracuel et al., 2012; Ogalla et al., 2015). El tratamiento con EESA fue capaz de compensar este remodelado vascular tanto en la dieta HFD como en la dieta LFD. Los ratones alimentados con la dieta HFD mostraron una menor rigidez asociada al incremento en la distensibilidad, lo cual redujo el estrés vascular, mientras que los valores de CSA se vieron reducidos en ambos tipos de dieta. Estas mejoras podrían estar relacionadas no solo con un efecto directo del EESA sobre el lecho mesentérico, sino también a consecuencia de la mejora en el desarrollo de la placa aterosclerótica en la aorta, la reducción del estrés oxidativo e inflamación vascular en aorta y arterias mesentérica, del perfil lipídico y de la deposición de colágeno. Estas mejoras fueron especialmente notables en el grupo alimentado con la dieta HFD suplementada con un 5% de EESA, lo que nos lleva a pensar que puedan estar detrás del menor remodelado observado en este grupo. Finalmente, las propiedades miogénicas dependen del contenido vascular relativo en colágeno (más rígido) y elastina (más flexible). A diferencia de la dieta LFD, la dieta HFD indujo un mayor depósito de elastina y colágeno, incrementando su rigidez y tensión. Si bien el tratamiento con EESA no modificó el contenido en elastina, el grupo cuya dieta se suplementó con un 5% de EESA experimentó una reducción en el contenido de colágeno, que podría explicar en parte la reducción de su CSA, rigidez y tensión vascular, mejorando la funcionalidad de estas arterias.

c) Esteatosis hepática

Un efecto secundario a la hiperlipidemia es el desarrollo de hígado graso no alcohólico, que contribuye al avance de la aterosclerosis mediante la liberación de factores aterogénicos como las VLDL, citoquinas proinflamatorias y PAI-1 (Edens et al., 2009).

Como era de esperar, los ratones ApoE-/- desarrollaron hígado graso. El tratamiento con EESA redujo esta acumulación de lípidos, siendo la dosis del 1% mucho más efectiva que la del 5%, que sólo mejoró la esteatosis en la dieta HFD. El **ácido ferúlico**, y uno de sus metabolitos, el ácido caféico pueden ser los responsables de esta mejora ya que su consumo está asociado a una menor síntesis hepática de triglicéridos y de la expresión de genes relacionados con el metabolismo hepático de triglicéridos y colesterol (FAS; DGAT-2, ATGL, PPAR-α, SREBP-2, LXR-α, LDL-R) (Bocco et al., 2016). Otro componente del EESA, los **fitosteroles** también se han asociado a una menor hiperlipidemia y esteatosis gracias a la inducción de una mayor excreción fecal de colesterol y a la normalización de la expresión de PPAR-α, CPT-1α y PCK-1en el hígado (Laos et al., 2014). En nuestro estudio, la dosis del 1% proporcionó una mayor protección frente al desarrollo de hígado graso independientemente de la dieta, a pesar de experimentar una menor bajada de colesterol plasmático en comparación con la dosis del 5%. Este hecho puede explicarse si tenemos en cuenta que la dosis del 5% proporciona un aporte superior de tocoferoles y tocotrienoles. Los tocotrienoles pueden biotransformarse en tocoferoles, que no sólo no tienen los mismos efectos antioxidantes y sobre la bajada del colesterol que los tocotrienoles, sino que también interfieren en su actividad cuando se consumen en grandes cantidades (Shibata et al., 2016). Además, los tocotrienoles tienen una especial preferencia a acumularse en tejidos grasos, como el tejido hepático. Finalmente, niveles plasmáticos elevados de las enzimas hepáticas aspartato aminotransferasa (AST) y alanina aminotransferasa (ALT) son indicativos de inflamación y daño hepático. En nuestro trabajo se observó un incremento de ambas enzimas con la dieta HFD, pero el tratamiento con EESA solo redujo los niveles de AST en la dieta HFD, que a diferencia de ALT no es específica del hígado, y puede reflejar procesos inflamatorios de otros tejidos, que si mejoraron en la dieta HFD a diferencia de en la dieta LFD.

d) Disfunción mitocondrial

Como se ha comentado anteriormente, la mitocondria es la principal fuente de ROS como subproducto de la cadena respiratoria a la vez que una diana directa para las modificaciones oxidativas. De hecho, la mitocondria requiere de mecanismos

antioxidantes locales altamente eficaces para neutralizar las ROS generadas. El estado proinflamatorio y de estrés oxidativo presente en la aterosclerosis puede acelerar el daño en el ADN mitocondrial, promoviendo su desacoplamiento y comprometiendo su correcto funcionamiento que derivará en la generación de enormes cantidades de ROS y estímulos proapotóticos, como la liberación del citocromo c o la proteína AIF (apoptosis inducing factor) al citoplasma, potenciando el desarrollo de la placa de ateroma (Arnoult, 2007). Dado que los procesos celulares requieren una síntesis eficaz de ATP, niveles bajos de biogénesis o un ciclo de vida mitocondrial alterados se consideran indicativos de un metabolismo y funcionalidad bioenergética celular alterados (Knott et al., 2008).

El regulador principal de la biogénesis es la proteína **PGC-1α** (cofactor 1α del receptor activado por el proliferador de peroxisomas), a través de la inducción de la expresión de los factores **NRF-1/2** (factor de respiración nuclear) y **TFAM** (factor de transcripción mitocondrial A), los cuales inducirán a su vez la expresión de proteínas necesarias para respiración mitocondrial (Wu et al., 1999).

Los ratones tratados con EESA 5% normalizaron la expresión de ARN mensajero de los principales marcadores de biogénesis mitocondrial (*Pgc-1α, Pgc-1β y Nrf-1*) en la aorta, mientras que *Tfma* no se vio modificado y *Nrf-2* no recuperó los niveles basales. La actividad y expresión de PGC-1α es controlada por AMPK, que promueve su expresión y reduce la acetilación inhibitoria de PGC-1α y por sirtuina 1, que activa a PGC-1α mediante fosforilación (Cantó et al., 2009). Aunque los niveles expresión de sirtuina 1 no se vieron modificados por el tratamiento con EESA, el incremento en la expresión y fosforilación de AMPK en el grupo tratado con EESA explicaría la mayor expresión y desacetilación de PGC-1α y por lo tanto de la recuperación de la biogénesis mitocondrial observada.

El efecto positivo del EESA y sus componentes mayoritarios sobre el estado de fosforilación de AMPK y su expresión, fue investigado *in vitro* en la línea de células endoteliales bovinas (BAEC), confirmando que una mayor fosforilación podría estar inducida por el **ácido ferúlico**. Otros autores han demostrado previamente que un metabolito del ácido ferúlico, el ácido caféico, ha mostrado el mismo efecto sobre la fosforilación de AMPK en líneas celulares de musculo liso (Eid et al., 2012), y tanto el ácido ferúlico como otros derivados del ácido hidroxicinámico consiguieron una mayor actividad de AMPK en células musculares y adipocitos (Bruckbauer et al., 2014). Por otra parte, la regulación diferente de la expresión los efectores NRF-1 y NRF-2 observada en este trabajo podría explicarse por el hecho de que, a diferencia de NRF-1, no existe una interacción directa entre PGC-1α y NRF-2, sino que la regulación de PGC-1α sobre NRF-2 podría estar

mediada por un tercer efector, que podría no estar regulado por el EESA (Gleyzer et al., 2005).

Los procesos de **fisión** mitocondrial son de vital importancia para la eliminación de los elementos mitocondriales dañados y el reciclaje de los aquellos que se mantienen intactos. Por otra parte, el mecanismo de **fusión** es especialmente importante en las primeras etapas de la biogénesis mitocondrial y tras la fisión, con objeto de aumentar el tamaño del orgánulo. No obstante, debe existir un **equilibrio** entre ambos procesos para asegurar **tamaño, número y forma** equilibrados para el correcto funcionamiento de las mitocondrias (Horbay et al., 2016).

En nuestro trabajo, el EESA consiguió restaurar la expresión de ARN mensajero de los marcadores de fusión ***Mfn1*** (mitofusina-1) y ***Mfn2*** (mitofusina-2) y de fisión ***Fis1*** *(*proteína de fusión mitocondrial 1*)*, garantizando el equilibrio del ciclo mitocondrial. Asimismo, el EESA restauró los niveles de ARNm de ***Beclin-1***, implicado en procesos de mitofagia, evitando así el acumulo de restos mitocondriales no funcionales. El mismo incremento sobre la expresión de ARNm de PGC-1α y MFN1 se observó en PBMC humanas tras el consumo de ácido ferúlico durante 15 días, lo que nos indica que las mejoras en el ciclo mitocondrial podrían estar relacionadas con un menor estado de estrés oxidativo y con la inducción de la actividad de AMPK, alcanzada en la aorta de los ratones ApoE-/- y en células BAEC tras el tratamiento con EESA.

Aunque no hemos comprobado si estas mejoras introducidas por la suplementación con EESA afectan directamente a la función mitocondrial en la aorta debido a dificultades técnicas para conseguir una cantidad adecuada de las mismas en este tejido, otros autores obtuvieron mejoras en la respiración mitocondrial así como en la cantidad de las mismas y su resistencia al estrés oxidativo en el cerebro de conejillos de indias a los cuales se administró un extracto de salvado de arroz durante 30 días (Hagl et al., 2013; Hagl et al., 2015). En estos trabajos también se atribuye a los efectos antioxidantes del extracto de salvado de arroz la actividad protectora del salvado de arroz, en especial al contenido en tocoferoles y tocotrienoles, que por su lipofilia atravesarían la barrera hematoencefálica y alcanzarían el cerebro, donde se acumularían. En nuestro caso, el consumo crónico de la dieta suplementada con EESA proporcionaría un aporte continuado de las principales moléculas antioxidantes presentes en el extracto, ácido ferúlico, tocoferoles y tocotrienoles, que en su transporte por el torrente sanguíneo proporcionarían la protección antioxidante necesaria para preservar la funcionalidad mitocondrial y el equilibrio de su ciclo de vida. De hecho, la sobreexpresión de la enzima antioxidante Mn-SOD (isoforma de SOD de localización mitocondrial) en la aorta de los animales

alimentados con la dieta HFD y la normalización tras la suplementación la dieta con EESA sería un indicativo de menor estrés oxidativo a nivel local en la mitocondria.

e) Apoptosis

La apoptosis juega un papel relevante en todos los estadios del avance de la aterosclerosis y su importancia para el progreso de la placa dependerá del estado de avance de la placa y del tipo celular implicado. La pérdida de células musculares puede tener un efecto negativo para la estabilidad de la placa, pues la mayoría de las fibras de colágeno, que son de crucial importancia para la resistencia a la tracción de la capa fibrosa, son producidas por las células musculares. Por su parte, una mayor apoptosis de células endoteliales llevará a una mayor activación del endotelio. Sin embargo, la apoptosis de los macrófagos puede tener un efecto beneficioso para la estabilidad de la placa siempre y cuando los cuerpos apoptoticos sean retirados del medio y no produzcan un núcleo necrótico, generando trombosis intra-placa o nuevos estímulos proinflamatorios (Kockx et al., 2000). Si bien la función principal de los macrófagos en la placa es la retirada de las oxLDL, que son reconocidas como un xenobiótico para el organismo, la presencia de macrófagos en la forma de células espumosas supone un estímulo proinflamatorio que potenciara el desarrollo de la placa.

En nuestro trabajo se observó una **regulación dual** de la apoptosis por parte del EESA, de manera que se vio reducida en la **aorta**, pero potenciada en los monocitos esplénicos. En monocitos esplénicos se observó una mayor expresión de p53 y de su efector bax. Entre otras funciones, p53 induce apoptosis de células con daños en el ADN que son irreparables, para evitar la proliferación de células defectuosas. Ante el estímulo proapoptotico, bax se une a la membrana de las mitocondrias, generando poros a través de los cuales se liberará el citocromo c que activará la vía de las caspasas. Por otra parte, la proteína bcl-2, presente en la membrana mitocondrial puede heterodimerizarse con bax, impidiendo su acción y manteniendo la integridad de la membrana, frenando así el proceso de apoptosis (Westphal et al., 2011). Sorprendentemente, la expresión de bcl-2 también aumentó en los **monocitos esplénicos** de los ratones tratados con EESA, hecho que podría explicarse como mecanismo compensatorio al incremento en la expresión de bax. Estudios de otros grupos han demostrado que componentes del salvado de arroz, como el γ-oryzanol, el ácido ferúlico, el β-sitosterol y especialmente los tocoferoles y tocotrienoles y el ácido fítico inducen la apoptosis e inhiben el crecimiento celular de diferentes líneas de cáncer, las cuales tienen una alta tasa de reproducción (al igual que los monocitos en la aterosclerosis), sin afectar a las células no malignas mediante la regulación de la expresión

de p53, bax y bcl-2 (Henderson et al., 2012; Al-Fatlawi et al., 2014). Este incremento en la apoptosis de los monocitos esplénicos podría ser una causa para su menor presencia en la placa de ateroma, reduciendo así el progreso de la misma en los animales tratados con EESA, como se ha sugerido previamente (Poch et al., 2004). Por el contrario, el EESA tuvo un efecto antiapoptótico en la aorta, con una menor expresión de p53 y bax, que podría estar relacionado con la protección antioxidante y antiinflamatoria proporcionada por el EESA. Estudios previos han demostrado el efecto protector de α-tocoferol y γ-tocoferol en la apoptosis inducida por oxLDL en células musculares lisas coronarias mediante el incremento en la expresión de bcl-2 y la reducción de la activación de la vía de las kinasas (MAPK y JunK) (de Nigris et al., 2000).

Por otra parte, tras el consumo de 500 mg de ácido ferúlico durante 15 días, las **PBMC humanas** mostraron una mayor resistencia a la apoptosis y necrosis inducida *ex vivo* tras su aislamiento con H_2O_2. En este caso, según sugiere el trabajo publicado por Khanduja et al. (2006), el mecanismo del ácido ferúlico sería independiente de bcl-2, y estaría relacionado con la capacidad antioxidante del ácido ferúlico y su protección sobre el daño al ADN.

f) Senescencia celular

El daño en el ADN y el acortamiento de **telómeros** son eventos relacionados con el desarrollo de enfermedades cardiovasculares, siendo un factor predictivo para el desarrollo de aterosclerosis (Fyhrquist et al., 2013). El alto grado de recambio celular que se da en las placas de ateroma puede dar lugar a un acortamiento de telómeros, que será mayor o menor en función de las características de la placa (Huzen et al., 2011).

En este trabajo, el EESA demostró una protección en la longitud de los telómeros de la aorta y monocitos esplénicos, por un mecanismo independiente de **telomerasa**, la enzima que replica el ADN de los telómeros, los cuales se ven reducidos tras cada ciclo de replicación celular. Este acortamiento de telómeros se ve acelerado por un elevado estrés oxidativo dado que el daño oxidativo es peor reparado en los telómeros que en el resto de material genético de los cromosomas (von Zglinicki, 2002). En la aorta, tanto los animales tratados con EESA como los que sólo consumieron la dieta HFD, expresaron una mayor cantidad de **TERT** (telomerase reverse transcriptase), la subunidad catalítica de la enzima telomerasa. Se ha descrito que la expresión de TERT puede ser activada por la vía proinflamatoria del NF-κβ (Gizard et al., 2011), típicamente activa en procesos de aterosclerosis. Sin embargo, en nuestro caso este incremento en la expresión de TERT no se

tradujo en un incremento de la actividad telomerasa. No obstante, tanto en la aorta como en monocitos esplénicos, se observó un incremento en la proteína **TRF2**. Esta proteína reconoce específicamente a los telómeros y los reorganiza en una estructura de bucle, protegiéndolos frente a la erosión oxidativa (Karlseder, 2003). Dado que tanto la expresión de TERT como TRF2 en la aorta se vio incrementada en los ratones ApoE-/-, independientemente de si consumieron EESA o no, la protección frente al acortamiento de telómeros del grupo de EESA debe atribuirse nuevamente a un menor estrés oxidativo presente en este grupo. Este menor daño en el ADN del grupo tratado con RBEE podría estar relacionado con la menor expresión de la proteína p16 en la aorta. La proteína p16 está asociada a la senescencia, ya que funciona como un secuestrador de la proteína Mdm2, responsable de la degradación de p53.

En el caso de los **monocitos esplénicos**, TRF2 sólo se incrementó en los ratones que recibieron el tratamiento con EESA. Esta mayor protección de los telómeros aunada a un menor daño oxidativo, explicaría que la actividad de telomerasa se viera también reducida ante una menor necesidad de reparación de los telómeros. Otra posible explicación es el efecto negativo que ejercen los tocotrienoles sobre la expresión de TERT mediante la inhibición de la vía de la protein kinasa C (Nakagawa et al., 2004; Eitsuka et al., 2006).

6. ***Biodisponibilidad del EESA***

Para la comprensión de los mecanismos moleculares del EESA resulta de especial interés el estudio de la biodisponibilidad de las principales moléculas bioactivas presentes en el extracto. Si bien gran parte de los efectos del salvado de arroz de han atribuido a la presencia de γ-oryzanol en su composición, se sabe que su absorción es muy baja o nula debido a su baja hidrosolubilidad, a la labilidad del enlace éster presente en su molécula y a su baja permeación a través de la barrera intestinal (Gillespie, 2003; Lubinus et al., 2013; Zhu et al., 2015). Sin embargo, otros estudios apuntan a procesos de hidrólisis previos a la liberación del ácido ferúlico y los diferentes tipos de fitosteroles que conforman la molécula de γ-oryzanol y que serán los verdaderos efectores de las acciones del γ-oryzanol (Berger et al., 2005; Mandak et al., 2012). Si bien la absorción de los fitosteroles es muy baja, el ácido ferúlico es capaz de alcanzar el torrente sanguíneo. Por ello nos propusimos investigar la absorción del ácido ferúlico y de sus metabolitos a través de la monitorización de sus principales metabolitos en sangre, orina, heces, así como su acumulación en tejido renal y hepático, como principales órganos encargados de su metabolismo y eliminación.

Tras la administración oral de EESA de forma aguda a ratas Wistar se encontraron un gran número de compuestos fenólicos en plasma, orina y heces. En **plasma**, la absorción de los compuestos fenólicos se ajustó a dos perfiles diferentes, respondiendo a los diferentes tiempos (t_{max}) necesarios para alcanzar los picos de concentración máxima (C_{max}). El primer grupo, con un t_{max} corto, presentó un segundo pico de concentración de menor magnitud y más tardío. Estos picos podrían explicarse por una primera absorción tras la administración del EESA (primer pico) seguida de una reabsorción derivada del ciclo enterohepático (segundo pico). El segundo grupo presentó un primer pico de menor extensión y un t_{max} tardío. Al igual que los compuestos del primer grupo, el primer pico se correspondería con la absorción tras la administración del EESA, mientras que el segundo pico se explicaría por el metabolismo de la microbiota a nivel de intestino grueso dando lugar a una mayor cantidad de estos compuestos en relación al primer pico **(Figura 24)**. En trabajos que describen la biodisponibilidad de derivados del ácido hidroxicinámico también encontraron metabolitos que pudieron clasificar en función de su t_{max}, como consecuencia de su absorción a nivel de intestino delgado o grueso tras fermentación por la microbiota (Rechner et al., 2001; Renouf et al., 2010; Stalmach et al., 2014).

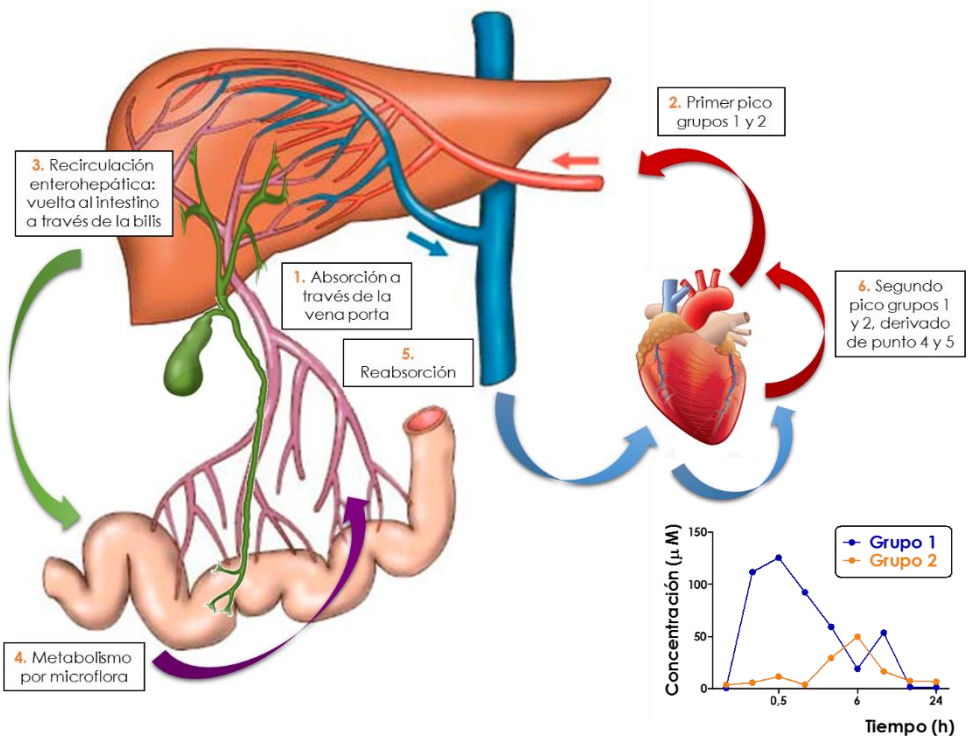


Figura 24: Perfiles plasmáticos de absorción de los compuestos fenólicos del EESA.

En nuestro trabajo, la mayoría de los metabolitos desaparecieron de plasma tras 24 h, indicando su rápido metabolismo, como ya había sido descrito para el ácido ferúlico (Fujiwara et al., 1982; Pan et al., 2014). Esta rápida eliminación explicaría el hecho de que no se encontraran trazas de ácido ferúlico en el suero de los ratones ApoE-/- tras 12 h de ayuno. Además, la rápida cinética de absorción del ácido ferúlico fue confirmada en **humanos** tras la administración de 500 mg de ácido ferúlico aislado. En este caso, se encontraron t_{max} y perfiles de absorción similares a los observados en las ratas Wistar tras la administración de EESA. Sin embargo, resulta interesante que algunos compuestos encontrados en las ratas Wistar (ácido cumárico, ácido homovainillínico, metilcatecol sulfato, catecol sulfato, ácido protocatecuico sulfato, ácido hidroxifenilácetico, ácido hipúrico, ácido fenilacético y ácido hidroxifenil-propiónico sulfato, así como algunos derivados de estas moléculas) no se encontraron en humanos, mientras que sólo dos moléculas del metabolismo en humanos (ácido dihidroferúlico glucurónido y ácido metoxibenzoico) no estaban presentes en el plasma de las ratas Wistar. Estas diferencias pueden deberse a diferencias metabólicas inter-especie o a que provengan de alguna otra molécula presente en el EESA y que no se suministró en los comprimidos de ácido ferúlico que consumieron los humanos.

La rápida metabolización del ácido ferúlico se vio confirmada por la aparición de los compuestos derivados del ácido ferúlico en **orina** en gran cantidad entre las 9 y las 48 h post-administración del EESA en las ratas Wistar, mientras que la fermentación bacteriana a nivel intestinal del ácido ferúlico y sus metabolitos se corroboró por la aparición de metabolitos sin conjugar en **heces** de los animales en el periodo de 24 a 48 h. Estos compuesto se forman a partir de metabolitos de fase II secretados a través de la bilis al intestino donde sufren la acción de enzimas bacterianas en los segmentos más distales del intestino, pudiendo ser reabsorbidos posteriormente cerrando el ciclo enterohepático.

Los metabolitos encontrados en las diferentes muestras biológicas sirvieron para proponer la **ruta metabólica** del ácido ferúlico. Los metabolitos detectados fueron fundamentalmente derivados azufrados, glucurónidos y metilados, fruto de la acción de enzimas sulfotransferasas, uridina-5'-difosfato glucuronosiltransferasas y catecol-O-metiltransferasas, siendo los derivados azufrados los más abundantes. Adicionalmente, los compuestos no absorbidos en el intestino delgado o los secretados por vía biliar podrían haber sufrido modificaciones por parte de la microflora previas a su absorción, fundamentalmente decarboxilación (α-oxidación) y dehidroxilación (ver figura 4 del capítulo VI). Finalmente, resulta interesante destacar que si bien la vida media del ácido ferúlico es corta, su acumulación en los tejidos responsables de su metabolismo y excreción

puede alargar su permanencia en el organismo, como se ha comprobado en hígado y riñones de los ratones ApoE-/- tras su consumo sostenido de las dietas suplementadas con EESA. Además, el ácido ferúlico interacciona con fitosteroles, de estructura muy parecida al colesterol, para dar lugar al γ-oryzanol. Resulta por tanto factible pensar que el ácido ferúlico pueda interaccionar con otras moléculas del organismo una vez absorbido. Esto es lo que describen varios autores que han encontrado interacciones entre el grupo ácido del ácido ferúlico y otros derivados del ácido hidroxicinámico con lípidos de membrana y con albumina sérica bovina (Ota et al., 2011; Jin et al., 2012). Estas interacciones, además de prolongar la permanencia de estas moléculas en el organismo, permitirían una protección antioxidante a nivel local, previniendo la peroxidación lipídica, que en el caso de los lípidos contenidos en las lipoproteínas, es de especial interés para prevenir la oxidación del colesterol.

7. Ensayos in vitro con los componentes mayoritarios del EESA

Con objeto de determinar la actividad antioxidante y antiinflamatoria de los compuestos bioactivos del EESA de manera aislada, se realizaron diferentes ensayos *in vitro* en cultivos de BAEC y PBMC y *ex vivo* sobre anillos de aorta.

a) Actividad antioxidante

La protección antioxidante del EESA y de sus compuestos aislados (ácido ferúlico, γ-oryzanol, β-sitosterol y γ-tocotrienol) frente al estrés oxidativo inducido por H_2O_2 fue probada en la línea de células endoteliales **BAEC**. El EESA fue capaz de prevenir el incremento de la peroxidación lipídica y la depleción de las reservas de glutatión. Este efecto fue mimetizado por el ácido ferúlico, mientras que γ-oryzanol y γ-tocotrienol mostraron efectos más moderados sobre la oxidación de glutatión y la peroxidación lipídica, respectivamente. Otros autores han demostrado el potencial antioxidante de productos derivados del salvado de arroz, como el aceite de salvado de arroz, y de los componentes aislados del salvado de arroz en modelos de aterosclerosis y en otras enfermedades cardiovasculares, identificando al γ-oryzanol, al ácido ferúlico y a los tocotrienoles como principales moléculas antioxidantes del salvado de arroz (Qureshi et al., 2001; Jin Son et al., 2010; Kwon et al., 2010; Masisi et al., 2016).

Los efectos inhibitorios del EESA y del ácido ferúlico y γ-oryzanol sobre la actividad de NADPHox se probaron *ex vivo* sobre anillos de **aorta** de rata Wistar, revelando al ácido ferúlico como responsable de la actividad antioxidante del EESA. La ausencia de efecto del γ-oryzanol *in vitro* puede deberse a que sea necesaria su hidrólisis, que ocurre en el

intestino por acción de esterasas pancreáticas, para liberar al verdadero efector que sería el ácido ferúlico. Además, la relevancia biológica del EESA sobre la inhibición de NADPHox se comprobó mediante la incubación de los anillos con suero de ratas que habían ingerido EESA y que se había caracterizado como rico en compuestos fenólicos, demostrando que tras la administración aguda de EESA se absorbe una cantidad suficiente y relevante para la inhibición de la actividad de NADPHox inducida por endotelina-1. Esta inhibición de NADPHox por parte del ácido ferúlico se corroboró además en **PBMC humanas** inducidas con H_2O_2.

b) Actividad antiinflamatoria

La inflamación al igual que el estrés oxidativo, están presentes en todas las etapas de desarrollo de la placa aterosclerótica en múltiples procesos que actúan de forma sinérgica. Los monocitos circulantes, precursores de los macrófagos en los tejidos, pueden clasificarse en tres subclases en base a la expresión de los receptores de superficie CD14 y CD16, que van a determinar sus características funcionales: monocitos clásicos (CD14++CD16-), monocitos no clásicos (CD14-CD16++), y monocitos intermedios (CD14++CD16+) (Ziegler-Heitbrock L et al., 2013). Ante un proceso inflamatorio, los monocitos clásicos e intermedios penetran en el tejido y se diferencian preferentemente a macrófagos M1, de perfil proinflamatorio, mientras que los monocitos no clásicos se diferenciarán preferentemente a macrófagos M2, de perfil antiinflamatorio **(Figura 25).** (Yang et al., 2014).

Monocitos humanos aislados inducidos con LPS fueron tratados *in vitro* con EESA, ácido ferúlico o γ-oryzanol. La inducción con LPS produjo un incremento en el porcentaje de monocitos intermedios en detrimento de los monocitos no clásicos, que se tradujo en un mayor viraje hacia macrófagos M1 y a una mayor producción de las citoquinas TNF-α e IL-6. El tratamiento con EESA tuvo un claro y fuerte efecto antiinflamatorio, con una mayor presencia de monocitos no clásicos y de macrófagos M2 y una reducción drástica de la liberación de TNF-α e IL-6. Si bien el efecto no alcanzó en todos los parámetros evaluados los niveles del EESA, tanto el ácido ferúlico como el γ-oryzanol mimetizaron su efectos. La potencia antiinflamatoria de γ-oryzanol y ácido ferúlico fue muy parecida, lo que nos lleva a pensar que los efectos antiinflamatorios están mediados por el ácido ferúlico. Por otra parte, el hecho de que el EESA presente un mayor efecto antiinflamatorio puede deberse a un efecto sinérgico de sus componentes, que actúen mediante diferentes mecanismos a varios niveles o que la interacción con alguno de los componentes presentes en el extracto, como las proteínas, favorezca su captación celular y absorción.

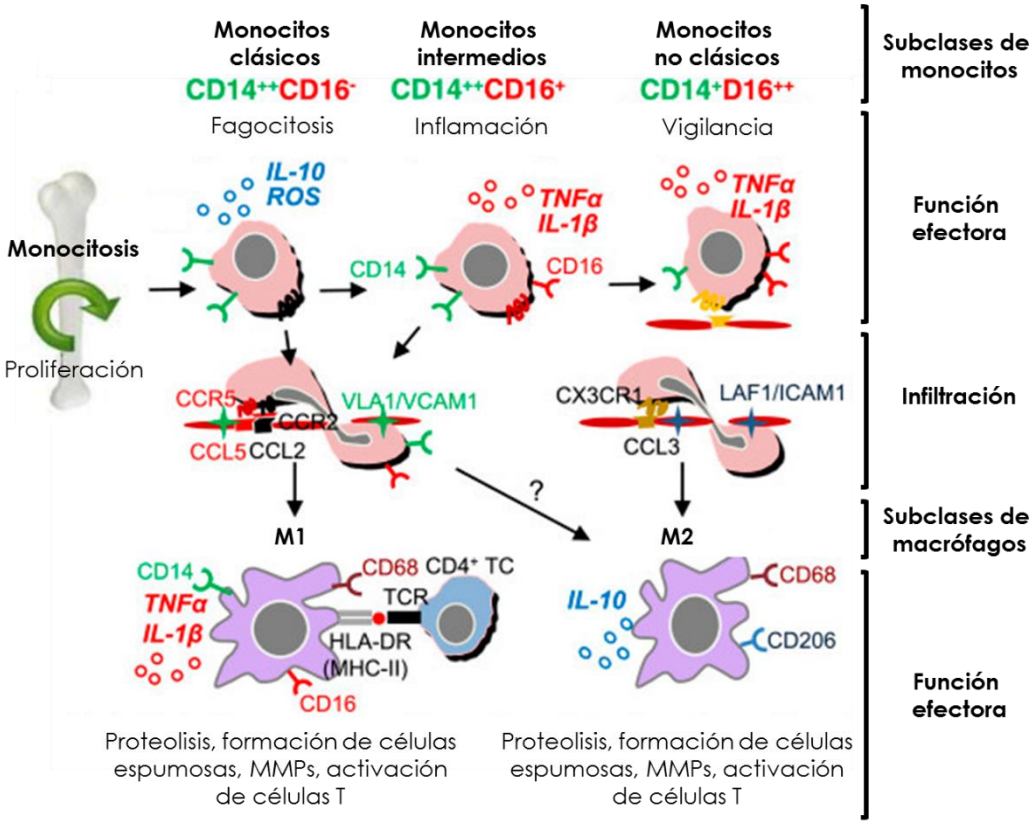


Figura 25: Subclases de macrófagos y monocitos. Traducido de Yang et al., 2014. Los monocitos clásicos presentan una alta expresión de CD14 y ausencia de CD16. Los monocitos no clásicos presentan una expresión muy baja de CD14 y altos niveles de CD16, mientras que los monocitos intermedios tienen una alta expresión de ambos receptores. Los monocitos clásicos tienen función fagocitaria, presentando una alta actividad peroxidasa y producen altos niveles de IL-10 ante un estímulo proinflamatorio. Los monocitos intermedios poseen actividad inflamatoria, con baja actividad peroxidasa, pero una elevada capacidad para liberar IL-1β y TNF-α. Ante un proceso inflamatorio, los monocitos clásicos e intermedios penetran en el tejido por interacción complementaria entre CCR2/CCL2 y/o CCR5/CCL5 y VLA1/VCAM1, mientras que los monocitos no clásicos penetran en los tejidos mediante interacción CX3CR1/CCL3 con LFA-1/ICAM-1.

PERSPECTIVAS FUTURAS

Los hábitos dietéticos de la sociedad actual son la causa de un incremento en la morbilidad y mortalidad por enfermedades cardiovasculares y cáncer. El abandono de la cardiosaludable dieta Mediterránea y su sustitución por otra donde predominan las grasas saturadas, el colesterol y los azúcares y están ausentes frutas y verduras, ricos en antioxidantes naturales, promueven la aparición de patologías como la aterosclerosis a edades muy tempranas que antes estaban asociadas al envejecimiento (Rodríguez Artalejo et al., 2002). Si bien se están perdiendo los hábitos dietéticos cardiosaludable, en los últimos años está surgiendo otra corriente que prima los **productos nutracéuticos** ricos en moléculas bioactivas como fuente de salud.

En este trabajo se ha demostrado el potencial del EESA para prevenir el avance de las consecuencias de una dieta alta en grasas y colesterol. Dados estos prometedores resultados, resultaría de gran interés realizar **ensayos clínicos** en los que se evaluara la capacidad hipolipemiante, antioxidante y antiinflamatoria del EESA, así como su impacto sobre desarrollo de aterosclerosis.

En vista de que los efectos de EESA sobre el control de la hipercolesterolemia fueron especialmente relevantes en la dieta alta en grasa y colesterol, el perfil de dieta occidental, rica en grasas saturadas y azucares, constituye un entorno ideal para la introducción de EESA en forma de suplemento nutracéutico o como parte de la composición de alimentos funcionales. Las **dosis diarias equivalentes** en humanos (HED), calculadas a partir del consumo de EESA por los ratones ApoE-/-, serían de **9.52 g** de EESA para la dosis del 1% y de **47.6 g** para la dosis del 5% al día para un peso medio de 70 kg (0.952 g y 4.76 si aplicamos el factor de seguridad). Estas cantidades podrían ser fácilmente introducidas en la dieta sin que supusiera un desbalance en su composición o aporte energético **(Figura 26)** (Sharma et al., 2009).

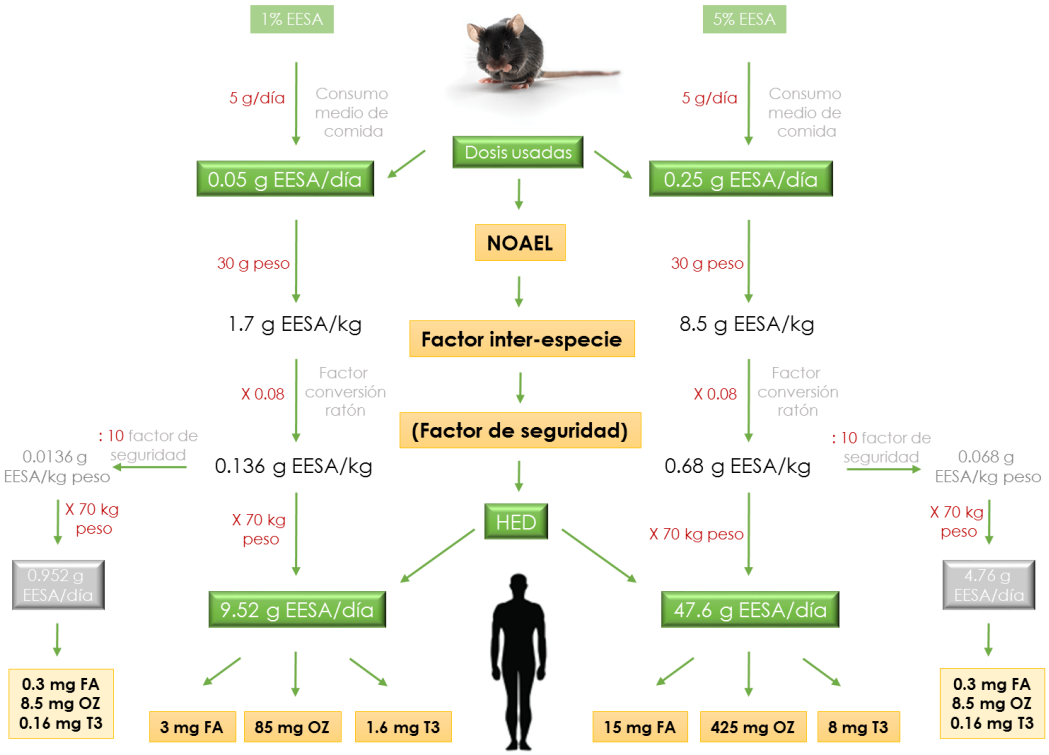


Figura 26: Cálculo de las dosis equivalentes en humanos (HED). A partir del consumo de EESA por los ratones ApoE-/- en función de su peso (g EESA/kg de peso corporal) obtendríamos el NOAEL (no observed adverse effect levels), que se corresponde con la cantidad del producto que no ha presentado efectos adversos. A continuación se aplica el factor específico inter-especie (0.08 en el caso de la conversión ratón-humano) y se obtienen la dosis de EESA equivalente para humano, a la cual se le podrá aplicar un factor de seguridad. A partir de la composición de EESA se pueden calcular las cantidades de los compuestos aislados ácido ferúlico (FA), γ-oryzanol (OZ) y tocotrienoles (T3).

El uso del salvado de arroz como materia prima para la elaboración de suplementos nutracéutico o alimentos funcionales resultaría de gran interés para la industria alimentaria. La alta producción de arroz en nuestro entorno podría abastecer las demandas de salvado de arroz, cuyo coste es además muy bajo por considerarse un desecho del proceso de refinado del arroz blanco. Las mejoras tecnológicas introducidas por la extracción enzimática facilitan la conservación y administración del EESA, eliminando las limitaciones de otras presentaciones clásicas de salvado de arroz, e incrementa el contenido en sus moléculas bioactivas, convirtiéndolo en una presentación idónea para su empleo en el control del desarrollo de la aterosclerosis.

CONCLUSIONES

Los resultados obtenidos en este trabajo sugieren que la suplementación de una dieta alta en grasas y colesterol con EESA supone una mejora frente al estado inflamatorio y de estrés oxidativo presentes en la aterosclerosis, previniendo su avance y el de otras alteraciones asociadas a la aterosclerosis: disfunción y remodelado vascular, esteatosis, apoptosis, senescencia celular y disfunción mitocondrial. Estos resultados nos permiten concluir lo siguiente:

1. La adición de EESA a la dieta alta en grasa y colesterol redujo la producción hepática de colesterol a través **HMG-CoA-Reductasa** e incrementó su **excreción fecal,** mejorando los niveles plasmáticos de colesterol total, triglicéridos y colesterol HDL. Sin embargo, la suplementación con EESA de la dieta baja en grasas no produjo ningún efecto, evidenciando la importancia del contenido graso y en colesterol de la dieta para el mecanismo de acción del EESA.

2. El desarrollo de la **aterosclerosis se redujo en válvula aórtica y cayado** de los animales alimentados con una dieta alta en grasas y colesterol suplementada con EESA. La menor aterogénesis en estos animales se relacionó con una menor hiperlipemia, la reducción del estrés oxidativo, de la deposición de lípidos y de partículas de LDL oxidadas en la aorta, así como la reducción del estado inflamatorio, de la expresión de las moléculas de adhesión VCAM-1 e ICAM-1 y de la infiltración de macrófagos en la placa.

3. En ratones ApoE-/- alimentados con una dieta alta en grasas y colesterol, los **mecanismos antioxidantes** del EESA a corto plazo son más eficaces en la dosis del 1% EESA y están relacionados con una menor actividad y expresión de NADPHox y con una mayor expresión de CuZn-SOD. A largo plazo, se observan mejoras en los niveles de oxidación del glutatión, peroxiredoxina, LDL y de la peroxidación lipídica con la dosis del 5% EESA, que recupera su potencial gracias a la reducción de los lípidos plasmáticos y del estado proinflamatorio. La falta de eficacia de la dosis del 5% a corto plazo puede deberse a el alto aporte de tocotrienoles que al biotransformarse en tocoferoles interfieren en la actividad de los primeros.

4. La suplementación de una dieta alta en grasas y colesterol con EESA redujo la **respuesta inflamatoria** en la aorta a través de la reducción de la expresión de TNF-

α y de la activación de NF-κβ, que se tradujo en una menor expresión de las enzimas proinflamatorias COX-2 e iNOS y en una menor producción de NO.

5. La suplementación de la dieta alta en grasas y colesterol con EESA mejoró la **función endotelial** de arterias mesentéricas mediante la reducción de la fosforilación inhibitoria de $eNOS^{thr1177}$, traduciéndose en un incremento de la biodisponibilidad de NO. La mejora en la relajación dependiente de endotelio en la aorta se produjo sólo en animales de 10 semanas, poniendo de manifiesto que el efecto protector del EESA sobre la función endotelial sólo es posible en ausencia de placa de ateroma.

6. El tratamiento de humanos con ácido ferúlico mejoró la proliferación y capacidad de migración de **células progenitoras endoteliales**, lo cual podría suponer un nuevo mecanismo del EESA de protección de la función endotelial.

7. La suplementación de las dietas alta y baja en grasas y colesterol con EESA previno la **remodelado de arterias mesentéricas** mediante la normalización de las características estructurales, miogénicas y mecánicas, estando estas mejoras relacionadas con la disminución de la deposición de colágeno, con la reducción del estrés oxidativo y con las mejoras derivadas de la reducción de la placa aterosclerótica en la aorta.

8. El tratamiento de ratones ApoE-/- con EESA redujo el desarrollo de **hígado graso no alcohólico**, siendo la dosis del 1% más efectiva que la del 5% EESA, pudiendo estar este hecho relacionado con el mayor aporte de tocotrienoles presentes en la dosis del 5% EESA.

9. La suplementación de la dieta alta en grasa con EESA al 5% normalizó la **biogénesis mitocondrial** a través la desacetilación activadora de PGC-1α mediada por AMPK, cuya fosforilación es inducida por EESA. La reducción del estrés oxidativo consiguió mantener el equilibrio en la expresión de los mediadores de los procesos de fusión (*Mfn1, Mfn2*) y fisión (*Fis1*) mitocondrial, así como de mitofagia (*Beclin-1*).

10. La suplementación de una dieta alta en grasas y colesterol con EESA 5% reguló de forma diferencial la **apoptosis** en la aorta y monocitos esplénicos de los ratones ApoE-/-. Suplementos de EESA previnieron la apoptosis en la aorta y endotelio vascular. En cambio, en monocitos esplénicos, el tratamiento con EESA potenció la apoptosis. Este incremento en la apoptosis de monocitos puede relacionarse con

la reducción de la infiltración de macrófagos en la placa aterosclerótica y su desarrollo.

11. La suplementación al 5% EESA de la dieta alta en grasas y colesterol previno el **acortamiento de telómeros** en la aorta y monocitos esplénicos de ratones ApoE-/- por un mecanismo independiente de la actividad de telomerasa. El incremento de la expresión de TRF2 en monocitos esplénicos y el menor estrés oxidativo en la aorta se sugieren como mecanismos responsables de esta protección.

12. Tras la absorción de ácido ferúlico puro o a partir de EESA se generaron una multitud de **compuestos fenólicos en plasma**, con diferentes perfiles de absorción fruto del metabolismo de la microflora intestinal y de la recirculación enterohepática. La **absorción, metabolismo y eliminación** del ácido ferúlico y sus metabolitos fue rápida pero suficiente como para mostrar actividad antioxidante tras su administración aguda o como consecuencia de su acumulo en los tejidos tras la administración sostenida.

13. En el **metabolismo del ácido ferúlico** se producen fundamentalmente metabolitos azufrados, glucurónidos y metilados, fruto de la acción de enzimas sulfotransferasas, uridina-5'-difosfato glucuronosiltransferasas y catecol-O-metiltransferasas. La aparición de metabolitos diferentes en humanos y ratas Wistar sugiere que el metabolismo pueda diferir según la especie o que el EESA puede ser fuente de otros compuestos fenólicos que no se obtendrían a través del consumo de ácido ferúlico aislado.

14. Estudios ***in vitro* con BAEC** inducidas con H_2O_2 y tratadas con los compuestos aislados del EESA identificaron al ácido ferúlico como principal responsable de la menor oxidación del glutatión y de la reducción de la peroxidación lipídica. Estudios *ex vivo* en anillos de aorta de rata con los compuestos aislados o con plasma de ratas enriquecido con los metabolitos biodisponibles del EESA confirmaron la actividad antioxidante del EESA mediante la inhibición de la enzima NADPH oxidasa.

15. El estudio ***in vitro* de monocitos humanos** puso de manifiesto que las mejoras en el estado proinflamatorio podrían deberse en parte al intervención del EESA, ácido ferúlico y γ-oryzanol en la polarización de monocitos y macrófagos, favoreciendo perfiles más antiinflamatorios.

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EVALUATION OF THE PROTECTIVE EFFECT OF A RICE BRAN ENZYMATIC EXTRACT AGAINST THE INFLAMMATORY STATE PRESENT IN THE ATHEROSCLEROTIC PROCESS

Memory presented by **Cristina Pérez Ternero** to opt for the degree of Doctor in Pharmacy, with International Mention

Supervisors

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INTRODUCTION

Rice bran is a phytochemical-rich layer that surrounds the endosperm of whole grain brown rice. It is produced as a by-product of white rice milling and discarded or designated to animal feeding due to its tendency to rancid reactions that impede its safe consumption (Da Silva et al., 2006). However, there is accumulating evidence that consumption of stabilized rice bran promotes health benefits in humans due to the multifactorial activities of its minor constituents (Islam et al., 2011). **γ-oryzanol**, the main bioactive molecule in the insaponificable fraction of rice bran, accounts for well recognised lipid lowering and antioxidant activities (Bhaskaragoud et al., 2016). γ-oryzanol is cleaved into **ferulic acid** and sterols by intestinal lipase enzymes. Sterols in the intestine promote cholesterol elimination by competing with cholesterol absorption, while ferulic acid is absorbed and inhibits hepatic cholesterol synthesis by 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA-Reductase) though downregulation of its activity and by reduction of sterol regulatory element-binding protein (SREBP) expression, which is necessary for HMG-CoA-Reductase synthesis (Wang et al., 2015; Naowaboot et al., 2016). Moreover, ferulic acid has been found to reduce oxidative stress by inducing a more favourable balance between the expression of enzymes related to oxidative stress, such as NADPHox, in favour of the antioxidant defences such as superoxide dismutase, catalase, glutathione peroxidase or glutathione reductase (Ohara et al., 2009; Kwon et al., 2010; Senaphan et al., 2015). Apart from γ-oryzanol and its constituent molecules, **tocotrienols** are the second group of molecules that play important roles in the lipid lowering and antioxidant activities of rice bran. The antioxidant activity is based on the free radical scavenging capacity, while the reduction of plasma cholesterol is due to the increased on HMGR degradation and the reduction on the efficacy of its RNA transcription (Parker et al., 1993). It is important to note that the side chain unsaturations present in tocotrienols, but not in tocopherols, allow better tissue distribution and determine the higher lipid lowering and antioxidant activity of tocotrienols. In addition to the lipid lowering and antioxidant activities, rice bran is known by its anti-inflammatory actions through reduction of NF-κβ activation (Akihisa et al., 2000; Nagasaka et al., 2007; Islam et al., 2008; Islam et al., 2009). Moreover, rice bran also has an important antidiabetic activity related to the normalization of liver enzymes implicated in the metabolism and synthesis of glucose (hepatic glucokinase, PEPCK, G6pase), and to increased secretion of insulin by the pancreas (Kim et al., 2010; Son et al., 2011; Ghatak et al., 2012; Kozuka et al., 2013).

Prior works of our group showed improvement of several markers of metabolic syndrome and obesity of animals whose diet was supplemented with a rice bran enzymatic extract (RBEE). The **enzymatic extraction** is carried out at controlled pH and temperature to preserve the activity of the phytochemicals, and allows the stabilization of the original raw material by inactivation of endogenous lipases. The final product is a water-soluble syrup with higher biological quality compared to raw rice bran. There is a net increase in the

bioactive components, mainly of γ-oryzanol and tocotrienols, and the fragmentation of the proteins into peptides of low molecular weight allows the solubilization of the hydrophobic components by interaction (Parrado et al, 2006). RBEE diet supplementation has shown cardiometabolic protection in several animal models. RBEE supplements to high fat diet improved total cholesterol and HDL cholesterol in Wistar rats (Revilla et al., 2009). Moreover, RBEE-supplemented diet was able to attenuate hypertension insulin resistance and to improve serum lipids in obese Zucker rats (Justo et al., 2013b). Moreover, vascular function of the aorta and mesenteric arteries of Zucker rats was improved by increasing the expression of eNOS and calcium activated potassium channels and decreasing vascular oxidative stress and inflammation (Justo et al., 2013a, Justo et al., 2014). Moreover, RBEE supplements attenuated structural changes of adipose tissue and normalized the expression of pro-inflammatory markers such as TNF-α, IL-6, IL-1β and iNOS and pro-inflammatory macrophage polarization of Zucker rats and diet induced obesity-mice (Candiracci et al., 2014; Justo et al., 2016).

These promising protective cardiometabolic activities derived from diet supplementation with RBEE are of great interest for atherogenesis prevention. Atherosclerosis is the underlying cause for 80% of the deaths caused by cardiovascular diseases. Atherosclerosis is characterized by a low-grade systemic inflammatory and prooxidant state (Libby, 2002), causing the narrowing of the arteries produced by the accumulation of cholesterol, collagen, vascular smooth muscular cells and other cell types in the vessel wall. These plaques can eventually break causing thrombosis that could block blood flow to tissues, precipitating ischemic events. The process is trigged by small endothelial damages that cause a change in the endothelium, incrementing its permeability to cholesterol and immune cells. Cumulative LDL cholesterol in the arterial wall and its oxidative modifications stimulates monocyte infiltration and foam cell growth by cholesterol phagocytosis. The process is enhanced by oxidative stress and inflammation, which are the underlying cause of other pathologies that come along and potentiate atherosclerosis.

Endothelial dysfunction is a predictive factor for atherosclerosis development. Hypercholesterolemia induces the activation of NADPHox reducing the amount of NO available for vasorelaxation, and reduces the activity of eNOS (Warnholtz et al., 2001; Davignon et al., 2004). In further stages of the progression of atherosclerosis, vascular remodelling due to plaque growth, VSMC proliferation and collagen deposition can alter vascular function, both in conductance as well as resistance vessels. Another consequence of hypercholesterolemia is the development of non-alcoholic fatty liver (Kim et al, 2014). However, as well as being a consequence, steatosis can also promote atherosclerosis through liberation of proinflammatory cytokines such as TNF-α and IL-6. These prooxidante

and proinflammatory stimuli increase vascular apoptosis causing a high cellular turnover that will lead to early cellular senescence, another cause of atherosclerosis promotion (Uryga et al., 2016). However, macrophage apoptosis could be beneficial to prevent foam cell formation as long as apoptotic bodies are removed and not accumulated in the plaque core (Kockx et al., 2000). Cellular apoptosis is also induced by dysfunctional mitochondria. Mitochondria are both, source and target of oxidative stress. Reactive oxygen species are key mediators of signalling pathways underlying vascular inflammation in atherosclerosis progression. High oxidative environment causes the modification of mitochondrial DNA leading to dysfunctional proteins and uncoupled mitochondria, which will produce vast amounts of superoxide, promoting atherogenesis (Chistiakov et al., 2012).

Antioxidant-rich foods, such as rice bran can help to abrogate oxidative stress and inflammation. However, to understand the underlying mechanisms and the biological relevance, it is of great importance to investigate the absorption and metabolism of the active molecules. Although γ-oryzanol is the major phytochemical in rice bran, its appearance in serum was never detected due to its low water solubility, the size of the molecule and more importantly, to pancreatic esterase activity in the intestine which leads to the release of ferulic acid (Andreason et al., 2001; Mandak et al., 2012).

Diet is the first basic preventive measure as well as the first approach to hyperlipidemia. Rice bran, rice bran oil and its main bioactive molecules have shown ability to reduce plasma cholesterol and the complications derived from hypercholesterolemia in several clinical studies (Cicero et al., 2001). Therefore, rice bran and its technological- and qualitatively-improved presentation, the RBEE, appear as promising functional food ingredients for the prevention and treatment of atherosclerosis and atherosclerosis-related disorders.

JUSTIFICATION AND OBJECTIVES

Rice bran contains an interesting variety of bioactive compounds, including ferulic acid, γ-oryzanol, tocopherols and tocotrienols, and phytosterols. These molecules have demonstrated their lipid-lowering, antiinflammatory and antioxidant properties, and are therefore of great interest for the prevention of atherosclerosis, among other cardiovascular diseases. However, this potential is being wasted due to rice bran tendency to fatty acids oxidation, which hinders its use by the food industry. The enzymatic extraction of rice bran results in a completely water-soluble product due to the interaction of the oily components with the low molecular weight proteins. The enzymatic process also inactivates the lipases responsible for the rancid reactions and increases the concentration of the bioactive components. Prior studies of our research group have revealed the potential of rice bran enzyme extract (RBEE) in multiple markers of metabolic syndrome and obesity. This potential include improvement of moderate hypertension and insulin resistance, reduction of endothelial dysfunction in the aorta and resistance arteries, and reduction of vascular and adipose tissue oxidative stress and inflammation.

Given the biological potential of rice bran for the prevention of cardiovascular diseases and the technological improvement and in the concentration of the bioactive ingredients introduced by the enzymatic extraction, the general objective of this thesis was the study of the effects of diet supplementation with the RBEE on the progression of atherosclerosis in a genetic model of hypercholesterolemia and atherosclerotic plaque development.

The specific objectives for this thesis were the following:

- ✓ To carry out a review of the cardiometabolic and vascular effects of rice bran known to date, as well as its derived preparations and main bioactive molecules.
- ✓ To study the effect of RBEE on atherogenesis.
- ✓ To study the hypocholesterolemic mechanism of RBEE.
- ✓ To study the effects of RBEE consumption on the mechanisms related to the atherosclerotic process: oxidative stress and inflammation.
- ✓ To study the effects of dietary supplementation with RBEE on endothelial dysfunction in the aorta and resistance arteries.
- ✓ To study the effects of RBEE consumption on the appearance of functional alterations resulting from the atherosclerotic process, such as steatosis, mitochondrial dysfunction, cellular senescence and apoptosis.

- ✓ To study the bioavailability of one of the major bioactive molecules of RBEE, ferulic acid.
- ✓ To identify the bioactive molecules present in the RBEE that are responsible for the observed effects.
- ✓ To study the antioxidant effects derived from the consumption of ferulic acid in human polymorphonuclear cells.

CONCLUSIONS

The results obtained in this thesis suggest that the supplementation of a high fat and high cholesterol diet with RBEE improves the pro-inflammatory and prooxidant state present in atherosclerosis, preventing its progress and other alterations associated with atherosclerosis: vascular dysfunction and remodelling, steatosis, apoptosis, cellular senescence and mitochondrial dysfunction. These results allow us to conclude the following:

1. The addition of RBEE to the high fat and high cholesterol diet reduced the **HMG-CoA-Reductase** hepatic production of cholesterol and increased **cholesterol fecal excretion**, improving the plasma levels of total cholesterol, triglycerides and HDL cholesterol. However, RBEE supplementation of the low fat diet did not show the same effects, evidencing the importance of dietary fat and cholesterol for the RBEE hypolipidemic mechanism.

2. **Atherogenesis** was reduced in the aortic sinus and arch of animals fed a high fats and high cholesterol diet supplemented with RBEE. The lower atherogenesis in these animals was associated with lower hyperlipidemia, reduction of oxidative stress and lower deposition of lipids and oxidized LDL particles in the aorta. The reduction of the inflammatory state, of the expression of adhesion molecules VCAM-1 and ICAM-1 and of the infiltration of macrophages on plaque also contributed to the atherogenesis reduction.

3. In ApoE-/- mice fed a high fat and high cholesterol diet, 1% RBEE dose is more effective in the short-term **antioxidant effects** by downregulation of NADPHox activity and expression and by CuZn-SOD upregulation. In a long-term basis, greater improvements in glutathione, peroxiredoxin and LDL oxidation as well as in the lipid peroxidation levels are observed with the 5% RBEE dose, which recovers its potential through hypolipidemic and anti-inflammatory activities. The lack of efficacy of the 5% dose in the short-term effects can be due to the high content in tocotrienols, which can be metabolized to tocopherols, which interfere in the activity of tocotrienols.

4. The supplementation of a high fat and high cholesterol diet with RBEE reduced the **proinflammatory** response in the aorta through TNF-α downregulation and reduction of the activation of NF-$\kappa\beta$, which resulted in a lower expression of the proinflammatory enzymes COX-2 and iNOS and in a lower production of NO.

5. RBEE supplementation of the high fat and high cholesterol diet improved **endothelial function** of mesenteric arteries by reducing the inhibitory phosphorylation of $eNOS^{thr1177}$, resulting in an increase of NO bioavailability. The improvement in endothelium-dependent relaxation in the aorta occurred only in 10-week-old animals, showing that the protective effect of RBEE on endothelial function is only possible in the absence of the atherosclerotic plaque.

6. Ferulic acid treatment of humans improved the proliferation and migration capacity of **endothelial progenitor cells**, which could suggest a new RBEE mechanism for endothelial function protection.

7. The supplementation of high and low fat and cholesterol diets with RBEE prevented **mesenteric arteries remodelling** by normalizing the structural, myogenic and mechanical parameters. These improvements were related to the decrease of the collagen deposition, to the reduction of the oxidative stress and with the improvements derived from the reduction of the atherosclerotic plaque in the aorta.

8. The treatment of ApoE-/- mice with RBEE reduced the development of **non-alcoholic fatty liver,** being the dose of 1% more effective than the 5% RBEE, which may be related to the greater contribution of tocotrienols present in the dose of 5% RBEE.

9. Supplementation of the high fat diet with 5% RBEE normalized mitochondrial **biogenesis** through AMPK-mediated activating deacetylation of PGC-1α. The reduction of oxidative stress was able to maintain the equilibrium in the expression of the mitochondrial fusion (*Mfn1, Mfn2*), fission (*Fis1*) and mitophagy (*Beclin-1*) mediators.

10. Supplementation of high fat and high cholesterol diet with 5% RBEE differentially regulated **apoptosis** in the aorta and splenic monocytes of ApoE-/- mice. RBEE supplements prevented apoptosis in the aorta and endothelium. On the other hand, in splenic monocytes, treatment with RBEE enhanced apoptosis. This increase in monocyte apoptosis may be related to the reduction of macrophage infiltration in atherosclerotic plaque and its development.

11. Supplementation of high fat and high cholesterol diet with 5% RBEE prevented telomere shortening in the aorta and splenic monocytes of ApoE-/- mice by a mechanism independent of telomerase activity. Increased expression of TRF2 in splenic monocytes and lower oxidative stress in the aorta are suggested as mechanisms responsible for this protection.

12. After the **absorption** of pure ferulic acid or RBEE, a wide variety of phenolic compounds were generated in plasma, with different absorption profiles resulting from the metabolism of intestinal microflora and enterohepatic recirculation. The absorption, metabolism and elimination of ferulic acid and its metabolites was rapid but sufficient to show antioxidant activity after the acute administration or as a consequence of ferulic acid metabolites accumulation in the tissues after sustained administration.

13. Because of **ferulic acid metabolism,** sulfur, glucuronides and methylated metabolites are produced, resulting from the action of sulfotransferase enzymes, uridine-5'-diphosphate glucuronosyltransferases and catechol-O-methyltransferases. The occurrence of different metabolites in humans and Wistar rats suggests that metabolism may differ depending on the species or that RBEE may be a source of other phenolic compounds that would not be obtained through the consumption of isolated ferulic acid.

14. ***In vitro* studies with BAEC** induced with H_2O_2 in the presence of isolated bioactive compounds from RBEE identified ferulic acid as the main compound responsible for the lower oxidation of glutathione and the reduction of lipid peroxidation. *Ex vivo* studies in rat aorta with the isolated compounds or with bioavailable metabolites-enriched plasma confirmed the antioxidant activity of RBEE by inhibition of the enzyme NADPH oxidase.

15. ***In vitro* studies of human monocytes** revealed that improvements in the proinflammatory state could be due in part to the intervention of RBEE, ferulic acid and γ-oryzanol in the polarization of monocytes and macrophages, favoring the anti-inflammatory profiles.

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Las ideas son como las estrellas,

no llegarás a tocarlas con las manos,

pero como el marinero en el desierto de las aguas,

las eliges como guía y si las sigues alcanzarás tu destino.

(Carl Schurz, 1829-1906)